HIGH TEMPERATURE STRESS AND FLOWERING IN *BRASSICA NAPUS* L.

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RESEARCH QUESTIONS

How does high temperature stress affect seed production in plants (especially *Brassica napus*)?

Can a transgenic approach be used to overcome the effect of high temperature stress on seed production?

ABSTRACT

High temperature stress (HTS) adversely affects reproduction in most plant species studied to date. HTS during flowering may result in an almost total inhibition of seed production in crop plants. Increasing our knowledge of the effects of HTS on seed production will aid the breeding of more thermotolerant crop plants and improve our understanding of the effects of stress on plants.

An investigation of the effects of both drought and high temperature stress on the yields of barley, canola, flax, durum and spring wheat in five locations in Saskatchewan over a 25-year period was performed using multivariate analysis. Higher temperatures during June and July, when the plants were flowering, were correlated with reductions in yields of all the crops studied (except barley in June). A positive correlation between yields and precipitation during May and the winter preceding the growing season was observed.

In growth chambers, *Brassica napus* silique and seed production were inhibited during a ramping HTS treatment. This was due to a decrease in pollen germinability rather than a reduction in the number of flowers produced. HTS also caused reductions in megagametophyte fertility and disrupted embryo and/or seed development.

Transgenic plants were developed to overcome the effects of HTS on seed production. Two DNA constructs, one with the *Arabidopsis thaliana LEAFY* (*AtLFY*) promoter controlling *A. thaliana HEAT SHOCK PROTEIN 101* (*AtHSP101*) ORF expression and another with the *AtHSP101* promoter controlling *AtLFY* ORF expression, were inserted into *B. napus*. Other DNA constructs were made, using the constitutively expressed Cauliflower Mosaic Virus *35S* or the synthetic *EntCup4* promoters to control

expression of the *AtHSP101* or *A. thaliana HEAT SHOCK TRANSCRIPTION FACTOR 3* (*AtHSF3*) ORFs. These constructs were inserted into both *B. napus* and *A. thaliana*. Transgenic plants were tested using a ramping temperature regime but were found not to have increased flower thermotolerance.

During the manufacture of the DNA constructs it was determined that, in *A. thaliana*, 573 bp of *AtHSP101* had been copied between Terminal Inverted Repeats of a *Mu*-Like Element (*MULE*). This fragment was named *HSP101B*. In some transgenic *B. napus* and *A. thaliana* lines, containing 2046 bp of the *HSP101B* upstream regulatory region controlling β-glucuronidase (GUS) expression, cold-inducible GUS expression was observed. Methylation may have a role in control of endogenous *HSP101B* transcription.

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1WHTS	One-week High Temperature Stress
2WHTS	Two Week High Temperature Stress
35S	Cauliflower Mosaic Virus *35S* promoter
5'UTR	5' Untranslated Region
ABRC	Arabidopsis Biological Resource Centre
BAC	Bacterial Artificial Chromosome
bp	base pair
bu	bushel
CaMV	Cauliflower Mosaic Virus
c.v.	cultivar
eco.	ecotype
g	gramme
GLM	General Linear Model
GUS	-Glucuronidase
ha	hectare
HPLO	*HSP101* promoter : *LFY* ORF gene construct
h / hr	hour
HSC	Heat Shock Cognate
HSE	Heat Shock Element
HSF (HSTF)	Heat Shock (Transcription) Factor
HSP	Heat Shock Protein
HTS	High Temperature Stress
kb	kilobase pair
L	litre
LFY	LEAFY gene or protein
LPHO	*LFY* promoter : *HSP101* ORF gene construct
LTRE	Low Temperature Response Element
LTS	Low Temperature Stress
MANOVA	Multivariate Analysis of Variance
E	microEinstein

µg	microgramme
µl	microlitre
µm	micrometre
mg	milligramme
mL	millilitre
mm	millimetre
min	minute
MMDT	Mean Maximum Daily Temperature
MW	molecular weight
MWM	Molecular Weight Marker
Mu	*Mutator* (transposon)
MULE	Mutator-Like Element
ng	nanogram
(3')nos	nopaline synthase (3') transcription terminator
NPTII	Neomycin PhosphoTransferase II
PCR	Polymerase Chain Reaction
RM	Rural Municipality
rRNA	ribosomal RNA
RuBisCo	Ribulose 1,5-Bisphosphate Carboxylase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
S	Svedberg
se	standard error
sHSC	small HSC
sHSP	small HSP
t	tonne
T_1	1st transformed generation
Ti	Tumour inducing plasmid from *Agrobacterium tumefaciens*
TIR	Terminal Inverted Repeat
UNIANOVA	Univariate Analysis of Variance
X-Gluc	5-chloro-4-bromo-3-indolyl-glucuronide

CHAPTER 1 LITERATURE REVIEW

1.1 INTRODUCTION

High temperature stress (HTS) detrimentally affects flowering and seed production in many species (Table 1.1). Effects on flower production or fertility lead directly to a reduction in seed production; therefore, an understanding of the effects of HTS on plant reproductive structures is necessary in order to prevent heat-induced losses in the latter. By understanding the effects of HTS on seed production we may be better able to develop HTS tolerant crops, change agronomic practices and improve our understanding of the effects of stress on the physiology of plants. The effects of HTS have been studied primarily in crop plants, meaning that the majority of the literature focuses on the role of the stressor on seed production (Table 1.1). HTS also interferes with the function of vegetative tissues in many plants (discussed below). Both vegetative tissue function and reproduction in non-crop plants are likely to be affected in a manner similar to those observed in crop plants. The effects of HTS on plants offer us a glimpse of the interface between environment and organism.

HTS adversely affects plant reproduction both worldwide and across species. Species indigenous to a particular environment are likely to have evolved reproductive mechanisms to mitigate the effects of HTS conditions experienced in their ecosystems. Crop plants, on the other hand, are often introduced species and so can be adversely affected by the environment. For example, seed production by wheat in India and Australia (Australian Journal of Plant Physiology, Volume 21, Issue 6, 1998) and canola/rapeseed in Canada (Nuttal et al., 1992) are adversely affected by HTS. Rapeseed probably originates from the Mediterranean region.

Improved yields might be obtained from crop plants experiencing HTS by developing thermotolerant cultivars or by changing agronomic practices. For example, *Brassica* species are only grown during the winter months or in the mountains in India, in part to avoid HTS (Raj et al., 2001). If global warming due to greenhouse gas

Table 1.1 Examples of crop species affected by HTS during reproduction.

Species	Tissue(s) affected	Temperature (day/night)	Reference(s)
Arachis hypogaea	Flowers	34, 42 or 48ºC for 6 days	(Craufurd et al., 2000; Prasad et al., 1999; Prasad et al., 2000)
Brassica napus	Flowers / seed development	Field studies	(Jefferson et al., 1987; Kirkland and Johnson, 2000; Morrison and Stewart, 2002; Nuttal et al., 1992)
	Flowers, silique and seed development	27 to 35ºC	(Angadi et al., 2000; Morrison, 1993; Polowick and Sawhney, 1987; Polowick and Sawhney, 1988)
Brassica oleracea	Inflorescence	35ºC, 1 week	(Björkman and Pearson, 1998)
Capsicum annuum	Pollen	32ºC/26ºC, 8 days	(Aloni et al., 2001)
Linum usitatissimum	Flowers / seed development	Various (usually 35ºC+)	(Cross, 2002; Cross et al., 2003; Dybing and Zimmerman, 1965; Gusta et al., 1997; Kraft et al., 1963)
Lycopersicon esculentum	Flowers (pollen and pistils)	Various (30ºC or 32ºC during day)	(Peet et al., 1997; Peet et al., 1998; Sato et al., 2000; Sato et al., 2002)
Triticum aestivum	Flowers / seed development	Field studies	(Chipanshi et al., 1999; Entz and Fowler, 1990; Ferris et al., 1998; McCaig, 1997; Porter and Gawith, 1999)
	Pollen and ovules	30ºC for 1 day or 30ºC/20ºC for 3 days	(Saini and Aspinall, 1982; Saini et al., 1983)
Zea mays	Flowers / seed development	Field studies (30ºC+)	(Baszczynski et al., 1985; Carlson, 1990; Cheikh and Jones, 1994; Ottaviano et al., 1991)
	Pollen or developing kernels	32ºC /26ºC 38ºC /32ºC	(Herrero and Johnson, 1980; Lin and Dickinson, 1984; Schoper et al., 1986; Schoper et al., 1987)

accumulation causes an increase in summer temperatures, as models predict, a decrease in yields from all crops might occur.

It has been estimated that a 1°C increase in average maximum temperature in July results in a 0.4 t/ha (~10%) decrease in canola yield in Saskatchewan (Nuttal et al., 1992). Reductions in yield due to increases in maximum temperature may have a significant impact on the yields of many, if not all, crop species in Canada as many plants are adversely affected by HTS in a similar manner to that observed by Nuttal et al. (1992 Table 1.1).

1.2 Changes in growing practices and cultivars to overcome the effects of HTS

Environmental stresses in otherwise normal agricultural conditions cause a reduction in yields from the maximum genetic potential of a crop. One of these environmental stresses, low water availability, affects ~45% of the continental land area of the USA(Boyer, 1982). Without sufficient water to maintain transpiration, leaf temperatures can rise above their optima for metabolism (Mahan and Upchurch, 1988; Upchurch and Mahan, 1988). Therefore, plants growing in areas with low water availability are more prone to heat stress. Even with irrigation, crop yields still fall well below the maximum yields recorded under ideal conditions at experimental farms(Boyer, 1982). Furthermore, Boyer (1982) estimated that only 10% of the agricultural land in the USA could be irrigated. Irrigation of 10% of the agricultural lands in Saskatchewan would be both impractical and expensive. Less than one percent (320 256 acres) of the total seeded area was irrigated in 1996 (Saskatchewan Agriculture and Food, 1998), potentially exposing plants on the majority of the cultivated land area to HTS.

To overcome HTS-induced losses in crop yield in Saskatchewan, new planting practices or new thermotolerant cultivars are required. For example, earlier planting of *Brassica napus* in Saskatchewan reduces the risk of HTS-induced yield loss as flowering is well underway by July, when high temperatures occur (q.v. Angadi et al., 2000; Nuttal et al., 1992). High temperatures during flowering caused greater reductions in fertility

compared to HTS occurring during vegetative growth or pod maturation in *B. napus* (Angadi et al., 2000; Morrison, 1993; Nuttal et al., 1992).

Earlier planting of *B. napus* and other crops has drawbacks, however. Environmental conditions in early in the growing season may delay planting or slow plant growth as occurred in the spring of 1999 when high precipitation levels (snow) delayed seeding in many parts of Saskatchewan. This delay increased the risk of exposing plants in flower to high temperatures during July. Furthermore, as all crops are adversely affected by HTS and seeding takes place over a 2-3 week period, the crops sown last will be at the greatest risk.

Fall-seeded crops can flower earlier than those seeded in spring. For example, fall planted canola may flower up to two weeks earlier than canola planted in spring (Anon, 1998). Fall planting is dependent on good winter snow cover for seed survival. In addition, fall seeding practices for different areas of the Province are still being established. In 1999 only 1.5-2.2% of the total area planted with canola across the Canadian Prairies was Fall-seeded (Anon, 1999). The development of canola cultivars with thermotolerant flowers appears to be a more plausible method of reducing the effects of HTS on yield, taking into account current seeding practices and potential environmental challenges early in the seeding season in Saskatchewan.

An understanding of the mechanisms of thermotolerance is required if the development of thermotolerant crop varieties is to proceed in a directed manner. Changes in plant architecture have the potential to improve thermotolerance. For example, changes in leaf size, shape and angle to stem can reduce the heating effects of direct insolation (Mahan et al., 1995). Changes in plant water relations are also potential targets, such as modified stomata number and distribution. Other targets for modification include changes in water potential, water allocation and/or transpiration rate.

The wheat variety Bethlehem is an example of how improvements in thermotolerance can be achieved by altering plant architecture (Blum, 1986). Bethlehem wheat was selected for improved yields in dry Israeli conditions and was coincidentally found to have a higher level of thermotolerance than other varieties. A larger proportion of the photosynthetic area of this variety is in the awns, compared with less

thermotolerant varieties. As awns maintain their carbon uptake during HTS, a higher ratio of these organs to leaves and glumes results in higher photosynthate production during heat stress. Improvements in carbon uptake during HTS could be made by selecting for plants with increased ratios of thermotolerant to heat sensitive organs. Plants with awn:leaf ratios are disadvantaged however, as awns have a lower photosynthetic efficiency than leaves during non-HTS periods (Blum, 1986).

Differences in thermotolerance between cultivars have been observed in wheat (Blum, 1986), maize (Ottaviano et al., 1991) cowpeas (Hall, 1993), beans, soybeans, potatoes and tomatoes (Chen et al., 1982). The thermotolerant cultivars have a higher yield when exposed to high temperatures compared with the less thermotolerant varieties. Although it is possible to breed for thermotolerant plant varieties, genetic engineering would speed this process. Furthermore, because transgenes can be inserted into many plant species, one that improves thermotolerance in one species could be readily used in another, thus reducing crop development time considerably. In order to achieve a directed approach to improved thermotolerance a better understanding of the effects of HTS on crop plants is required.

1.3 THERMOTOLERANCE

All organisms have an optimal temperature range for growth and reproduction (Levitt, 1972). Temperatures beyond the optimal range cause stress and at further extremes become lethal. Organisms are generally able to withstand temperatures 5-10°C above the optimal growth temperature without being stressed. Acute high temperatures (15°C or more above the optimal range) are lethal depending on the speed of onset and duration of exposure (for examples see Lin et al., 1984). The damage caused by HTS is probably due to a combination of cellular changes (see below) and an inability of the cells to resume normal cellular, biochemical and physiological functions after return to normal temperatures.

Much work investigating thermotolerance in various organisms has focused on the "heat shock" response which is induced when cells or organisms are suddenly exposed to lethal or near-lethal temperatures. The elevated temperatures bring about rapid physiological changes in cells; however, such extremes rarely occur in nature. For

example, plants rarely experience 45° - 50°C temperatures for several hours in succession in nature. HTS, on the other hand is more likely to occur in the natural environment of an organism, e.g., an increase in temperature from 23°C to 35°C over several hours.

It is difficult to understand fully the processes involved in plant thermotolerance as HTS has multiple, broad ranging effects (see below). Breeding has increased thermotolerance in wheat (Blum, 1986; Viswanathan and Khanna-Chopra, 1996) and maize (Ottaviano et al., 1991) although the physiological changes behind these improvements are poorly understood. Furthermore, the polygenic nature of thermotolerance (q.v. Frova and Gorla, 1993; Ottaviano et al., 1991) increases the difficulty of breeding and maintaining favorable alleles in one cultivar. One analysis of recombinant inbred maize lines predicted that at least six loci were responsible for only ~40% of the observed differences in thermotolerance (Ottaviano et al., 1991).

An added difficulty is the differential thermotolerance of tissues within a plant. For example, awns (Blum, 1986) and fruit (Ferguson et al., 1998) have higher levels of thermotolerance than other tissues in wheat and apple, respectively. Wheat pollen tubes and ovaries (Saini et al., 1983), maize kernels during development (Cheikh and Jones, 1994), soybean root cells (Mansfield et al., 1988) and developing *B. napus* flowers (Morrison, 1993; Polowick and Sawhney, 1987; Polowick and Sawhney, 1988) are all sensitive to high temperatures. To date, investigations into the changes in plants brought about by high temperatures have focused at the cellular or tissue level.

1.4 Induced thermotolerance and cross-tolerance

Plants are able to adapt to an otherwise lethal HTS if they are first conditioned by a nonlethal temperature (Levitt, 1972). For example, *Sorghum bicolor* seedlings grown at control temperatures of 35°C were killed by a heat shock of 50°C for 2 hours. The seedlings develop a measure of thermotolerance if pretreated at 45°C for 2 hours prior to the heat shock, however. Induced thermotolerance of whole plants has been observed in many species including soybean (Lin et al., 1984), maize (Baszczynski et al., 1985), pea (Nagao et al., 1986) and cowpea (Hall, 1993). Thermotolerance has also been observed in lily and tobacco pollen (Herpen et al., 1989), soybean roots (Mansfield et al., 1988)

and organelles such as pea chloroplasts (Süss and Yordanov, 1986; Suzuki et al., 1998) and mitochondria (Schmitt et al., 1995).

How the physiology and biochemistry of a plant adapt to the changes in cellular function arising from a sublethal HTS are not well understood. Sublethal HTS induces a system to protect cellular proteins from irreversible denaturation as well as the synthesis of thermoprotective compounds in preparation for the eventuality of further HTS (see "Heatshock proteins" below Nguyen et al., 1989; Parsell et al., 1994; Schirmer et al., 1994). The rate at which these protective systems are induced by the sublethal or preconditioning HTS may be indicative of the ability of the plant to withstand a subsequent, otherwise lethal stress (Chen et al., 1982).

Thermotolerance can also be induced by other conditioning stressors, such as ethanol (Nguyen et al., 1989), arsenate (Key et al., 1985), heavy metal, drought (Bonham-Smith et al., 1987) and reactive oxygen intermediates (Fujita et al., 1998; Parsell and Lindquist, 1993). This phenomenon has been termed cross-tolerance or cross adaptation (Boussiba et al., 1975; Kampinga et al., 1995; Sabehat et al., 1998). Cross adaptation can also operate in the opposite direction; cells can be protected from ethanol, arsenate and oxidative stress by conditioning with a high temperature pretreatment.

1.5 HTS-INDUCED CHANGES IN CELL STRUCTURE AND FUNCTION

1.5.1 Changes in cell structure as a result of nonlethal HTS

Several changes were seen in soybean seedling root cortex cells preconditioned with a sublethal heat shock of 40°C for 3 hours (Mansfield et al., 1988). Changes included fusion of several smaller vacuoles into a single large one and the appearance of electron dense aggregates both in the mitochondria and appressed to the cytoplasmic side of the tonoplast. Cellular disruption was not observed in seedlings preconditioned at 40°C then subjected to lethal heatshock at 45°C for one hour. Cell lysis was common at 45°C in seedlings that had not been preconditioned.

Electron dense aggregates in the cytoplasm may be caused precipitation of denatured or damaged proteins (Fujita et al., 1998; Kampinga et al., 1995; Levitt, 1972).

Proteins denatured by HTS are dysfunctional and incur additional energetic costs to a cell through their disposal or reactivation once the HTS is removed.

1.5.2 Loss of control over membrane permeability

HTS causes alterations in lipid bilayer fluidity and membrane integrity, changing ion transport between cellular compartments. The changes in ion transport result in a loss of solutes and water and may disrupt membrane potential. Denaturation and subunit dissociation of membrane associated proteins may also result in changes to ion transport across membranes. Other HTS-induced changes to proteins may result in dissociation of proteins from the lipid bilayer or loss of ion selectivity by ion transport proteins.

The rate of ion leakage from stressed cells is often used as an indicator of plant thermotolerance. Thermotolerant varieties have lower rates of electrolyte loss than sensitive ones (Chen et al., 1982; Liu et al., 2000)

1.5.3 Changes in photosynthesis

The photosynthetic apparatus is highly susceptible to HTS (Berry and Bjorkman, 1980; Jiang and Huang, 2001; Liu et al., 2000). Normal protein expression is inhibited during heat shock (see below) including production of both photosystems and photosynthetic electron transport chain components (Süss and Yordanov, 1986). Furthermore, as HTS can alter membrane properties, proton and electron transfer via the photosynthetic electron transport apparatus is also affected (Jiang and Huang, 2001; Liu et al., 2000; Onwueme, 1979).

As temperatures rise, so does the rate of respiration. If respiratory demand exceeds photosynthetic supply, depletion of carbohydrate reserves occurs. The point at which this happens is the temperature compensation point, which is usually 3-12°C below the lethal temperature (Levitt, 1972). Extended periods above the temperature compensation point will severely deplete carbohydrate reserves stored in a plant.

A thermotolerant genotype of cowpea had a higher level of soluble sugars in the peduncles than heat sensitive genotypes during heat stress (Hall, 1993). This genotype consistently produced higher seed yields than other cowpea genotypes. Furthermore, yields from this cultivar did not change when the plants were placed under heat stress.

The higher degree of thermotolerance was probably due to improved stability of the photosynthetic apparatus. Growing the plants at both elevated CO_2 concentrations and temperatures increased the concentration of soluble sugars in the peduncle and improved the seed yield by almost 50%, compared to control plants. This indicated that the rate-limiting stage of photosynthesis during HTS was the influence of oxygen on RuBisCo activity, rather than reductions in the photoproduction of ATP.

HTS also inhibits RuBisCo synthesis in thermosensitive plants (Süss and Yordanov, 1986). *Phaseolus vulgaris* chloroplasts treated at 50°C contained denatured large subunits of RuBisCo. Upon return to normal growth temperature (25°C) only low levels of RuBisCo activity were restored indicating that, after HTS treatment, restoration of enzymatic activity to control levels requires *de novo* synthesis of the enzyme complex.

1.5.4 Changes in metabolite production

Plants under HTS produce lower amounts of sugars (photosynthesis is inhibited) and a reduced set of proteins, thereby limiting the synthesis of other products. In some drought or heat stressed cells, changes in membrane potential or reduced water availability can be compensated for by the synthesis of osmoprotectants like proline and glycine betaine. These compounds act as an osmotic solutes and increase osmotic potential in cells (Verma et al., 1993). How these osmolytes protect cells from water deficit and high temperature stress is not known in detail, but they may replace water molecules associated with proteins, thereby maintaining protein integrity and releasing scarce water molecules for other functions in the cell.

Other thermoprotective molecules found in plants include the sugar, trehalose (Newman et al., 1993), and isoprene (Singsaas et al., 1997). Trehalose is produced in *Saccharomyces cereviseae* and some higher plants during water deficit and heat stress (Newman et al., 1993). High concentrations of trehalose may stabilize protein structures by replacing water molecules associated with more hydrophobic amino acid residues. The released water molecules then associate with the more hydrophilic amino acid residues, helping preserve protein conformation. Trehalose may also replace water

bound to the hydrophilic heads of triacylglycerides, releasing these molecules from cell membranes and allowing them to bind to proteins.

Some plants, such as white oak and kudzu, secrete isoprene when tolerating a HTS (Singsaas et al., 1997). It is not known how isoprene functions to provide thermotolerance.

1.5.5 Protein aggregation and enzyme inactivation.

High temperatures directly cause changes in protein conformation resulting in denaturation and dysfunction. HTS-induced changes to protein structure may result from changing ion concentrations, pHs and ATP levels, all of which can result in further changes in the structures of proteins. Altered protein conformation can result in the exposure of hydrophobic amino acid residues to the aqueous cytoplasm; or in the case of membrane bound proteins, the exposure of hydrophilic amino acid residues to one another. Protein aggregation may occur if exposed hydrophobic residues in different proteins interact with one another.

Denatured proteins have lowered solubilities, leading to aggregation and formation of insoluble precipitates both *in vitro* and *in vivo* (Fisher et al., 1989; Fujita et al., 1998; Nguyen et al., 1989; Parsell and Lindquist, 1993). In *Drosophila*, heat shock (and other stressors) causes RNA polymerase II to aggregate in the insoluble nuclear matrix-pore complex lamina (Fisher et al., 1989), resulting in decreased transcription.

The inactivation of enzymes during heat stress has been studied using *E. coli* β-galactosidase and *Photinus pyralis* luciferase expressed in transgenic mouse and *Drosophila* cells (Nguyen et al., 1989). These foreign enzymes aggregated in the cytoplasm during heat shock and remained in this state even after the cells were returned to normal temperatures. Pretreatment of the transgenic mouse cells at 45°C for 15 minutes increased the half-life of β-galactosidase 10-fold and luciferase 2-fold during a subsequent 45°C heat shock administered twenty hours later. The 15-minute pretreatment also improved the ability of the cells to withstand subsequent ethanol exposure (cross-tolerance).

Inactivation of protein synthesis has also been observed in the chloroplasts of heat shocked *Phaseolus vulgaris* (Süss and Yordanov, 1986). Plants adapted to higher

temperatures were able to reverse protein inactivation once the heat stress was removed, however.

1.5.6 Genomic rearrangements

Recently it has become clear that abiotic stresses, including HTS, can cause genomic rearrangements. Cells exposed to stress have altered methylation patterns that alter the arrangement of heterochromatin, resulting in changes in gene expression (Ivashuta et al., 2002; Steward et al., 2000). Furthermore, the demethylation of transposons can activate transposase expression, resulting in the transposition of both autonomous and nonautonomous mobile genetic elements (Bennetzen, 2000). Transposon-mediated genomic rearrangements can have a variety of effects such as gene activation or deactivation, capture of a portion of a gene and co-suppression of gene activity. Genomic rearrangements resulting from stress may be a major source of genetic variation and populations living under stressful conditions may be genetically diverse. These mutations may not be observed phenotypically due to the action of HSP90, which is able to compensate for some changes in protein structure (Queitsch et al., 2002). *Drosophila* and *Arabidopsis* with compromised HSP90 function show a variety of different phenotypes resulting from genomic rearrangements (see "HSP90" below). Increased genetic diversity arising from stress may not be observed until sexual recombination brings together two mutant alleles or further stress overwhelms the capacity of HSP90 function to correctly fold proteins with major aberrations (Queitsch et al., 2002).

1.5.7 Changes in patterns of gene expression.

In plants, characteristic changes in the pattern of gene expression occur during HTS. A decrease in normal protein synthesis is observed with a concomitant increase in heat-inducible proteins (Brodl et al., 1990; Key et al., 1985; Levitt, 1972; Nagao et al., 1986). The reduction in protein synthesis is brought about by a breakdown in transcription and translation. For example., RNA polymerase II aggregates in heat-stressed *Drosophila* cells (Fisher et al., 1989). In addition, enzymes function less efficiently outside their optimal temperature range (Mahan and Upchurch, 1988) due to

changes in their structure and solubility (Nguyen et al., 1989). HTS could also cause disruptions in transcription factor conformation, resulting in altered transcription of genes and regulatory feedback mechanisms

The proteins induced by and synthesized during a heat shock are called heat shock proteins (HSPs). Their sizes fall within discrete molecular weight groups; 15-30 kD, 60-70 kD, 90 kD and 100-110 kD (Key *et al.*, 1990; Vierling, 1990). In plants, high levels of small HSPs (sHSPs) are produced during HTS (Waters et al., 1996) while the other classes of HSPs are produced at lower levels (Key et al., 1985; Vierling, 1990). The protein synthesis patterns are reproducible over a range of heat shock temperatures, and until cell death.

1.6 Heat Shock Proteins

The characteristic induced thermotolerance response, where a plant survives otherwise lethal high temperatures if pretreated with a sublethal heat stress, has been known for some time (Levitt, 1972). In the 1980's it was determined that HSPs produced during a sublethal pretreatment provided increased thermotolerance against the subsequent heat stress (Nagao et al., 1986; Vierling, 1990). During pretreatment, normal protein synthesis is inhibited and HSP are transcribed and translated rapidly in large amounts.

Exposure of plants to high temperatures induces the synthesis of abundant amounts of sHSPs (Waters et al., 1996) as well as HSP70s (DeRocher and Vierling, 1995; Li et al., 1999; Nagao et al., 1986; Roberts and Key, 1991; Sung et al., 2001) and HSP100s (Hong et al., 2000; Hong et al., 2001; Queitsch et al., 2000). Chloroplasts produce sHSPs, HSP60s and large HSPs (90-110 kD in size) when heat stressed (Süss and Yordanov, 1986).

The HSPs produced during HTS remain in the cell during the heat stress and are then degraded over the space of several hours after the removal of the high temperatures. Cells exposed to another heat shock during this period are protected from the effects of the high temperatures by the HSPs present and ones induced by this subsequent HTS. Organisms deficient in one or more HSPs are much more sensitive to heat shock, recovering slowly if at all (Parsell et al., 1994), while those overexpressing HSPs have

greater thermotolerance. For example, *S. cereviseae* cells deficient in one or more HSPs were less thermotolerant than wild-type strains (Parsell et al., 1994; Schirmer et al., 1994). Interestingly, complementation of yeast HSP mutations occurred with an orthologous plant gene, partially restoring thermotolerance. *Arabidopsis* plants with mutations in *HSP101* are also less thermotolerant than wild-type plants (Hong et al., 2000; Hong et al., 2001; Queitsch et al., 2000). Transgenic *A. thaliana* plants constitutively overexpressing a tobacco heat shock transcription factor (HSTF or HSF), which induces HSP transcription, showed an increased leaf and seedling thermotolerance of 2°C without the need for a pretreatment (Lee and Schöffl, 1996; Prändl et al., 1998). These observations indicate that the presence of HSPs are essential for thermotolerance.

Cross-tolerance to a stress results as the HSPs from the preconditioning stress are still in the cell (Sabehat et al., 1998). HSPs protect cellular proteins from irreversible denaturation, such as those occurring in cells exposed to HTS, ethanol toxicity (Nguyen et al., 1989), arsenate (Key et al., 1985), heavy metal (Bonham-Smith et al., 1987) and oxidative stress (Parsell and Lindquist, 1993). When ethanol, arsenate and hydrogen peroxide induce HSP expression, the cells have an increased thermotolerance to subsequent high temperatures (Kampinga et al., 1995).

Patterns of HSP expression in plants vary between tissues (Cooper et al., 1984; Dupuis and Dumas, 1990; Hernandez and Vierling, 1993; Jinn et al., 1997; Sung et al., 2001), organelles (Lee et al., 1995a; Nover and Scharf, 1997; Süss and Yordanov, 1986; Suzuki et al., 1998) and developmental stages (DeRocher and Vierling, 1995; Young et al., 2001). Maize and pea seeds and embryos produced large amounts of HSPs during normal development (Cheikh and Jones, 1994; DeRocher and Vierling, 1995) as did the microspores of various plant species (Atkinson et al., 1993; Duck and Folk, 1994; Gagliardi et al., 1995; Marrs et al., 1993; Mascarenhas and Crone, 1996). Constitutively expressed HSPs are sometimes termed heat shock cognates (HSCs) to differentiate them from their heat-induced counterparts.

In the field, HSP synthesis has been observed in soybeans (Kimpel and Key, 1985) and apple fruit (Ferguson et al., 1998). mRNAs corresponding to small MW HSPs (sHSP) were produced in the leaves of field-grown soybeans during heat stress (40°C

canopy temperatures), thereby matching HSP expression previously observed in growth chamber-grown plants (Kimpel and Key, 1985). In field-grown soybean plants, mRNA for three sHSPs were not detected the morning following a heat stress indicating that sHSP mRNA synthesis did not continue during the lower night temperatures. Irrigated plants exposed to high daytime temperatures (39°C) always had lower levels of the sHSP mRNAs examined than non-irrigated plants in an adjacent field, at various time points during the day (Kimpel and Key, 1985). Transpirational cooling, or greater water availability to maintain protein structure, may explain the reduced need for HSPs in the irrigated plants.

Diurnal patterns of HSP mRNA and protein expression were observed in apple fruit (Ferguson et al., 1998) indicating that light may play a role in the induction of these proteins. Tomato plants kept in the dark were found to be less thermotolerant than those grown in constant light. Re-illumination of dark-grown plants restored their ability to mount a thermotolerance response (Adelaja and Onwueme, 1993).

1.7 HSP FUNCTION

Both constitutively expressed HSCs and heat-inducible HSPs from all the HSP classes are involved in protein folding (Buchner, 1999; Hartl, 1996; Lee et al., 1995a; Nguyen et al., 1989; Nover and Scharf, 1997; Parsell et al., 1994). HSPs prevent protein denaturation during HTS and allow their renaturation after removal of the heat shock. Spontaneous renaturation of denatured proteins after high temperatures occurs rarely and at a slow rate, so refolding is facilitated by the HSP folding machinery (Parsell et al., 1994). HSCs are thought to be involved in the folding of *de novo* synthesized proteins.

Some HSPs are also classified as "chaperonins", proteins that guide the development of secondary and tertiary structure in proteins *de novo* synthesized during non-stress periods and the refolding of proteins denatured during stress (Buchner, 1999; Glover and Lindquist, 1998; Li et al., 1999).

As heat shock proteins are highly conserved throughout all taxonomic kingdoms they are thought to play an essential roles in cellular function (for reviews of HSP expression in plants see Agarwal et al., 2002; Katiyar-Agarwal et al., 2001; Nagao et al., 1986; Parsell and Lindquist, 1993; Vierling, 1990; Viswanathan and Khanna-Chopra,

1996). The high level of amino acid sequence conservation and the occurrence of orthologous genes across species means that transgenic HSPs are able to function when expressed in different cellular backgrounds. For example, soybean and *Arabidopsis HSP101* complement *S. cereviseae* cells with mutations in the orthologous *HSP104* gene (Lee et al., 1994; Schirmer et al., 1994).

Comparisons between the *S. cereviseae* proteins HSP104, YDJ1 (in the HSP40 Class) and SSA1 (a HSP70) with their corresponding plant proteins showed high levels of identity and conserved amino acid substitutions (personal analysis). *S. cereviseae* HSP104 and YDJ1 share approximately 45% identical and 60% conserved amino acid residues with orthologous proteins from various plants. SSA1 shares 71% identity and 83% conserved amino acid residues with pea HSP70.1. Of these chaperonins only *HSP101* has been shown to complement *S. cereviseae hsp104* mutants. Taking into account the high level of amino acid conservation, I would expect the plant orthologues to complement yeast *ydj1* and *ssa1* mutants.

B. napus plants and bromegrass cells treated with 24-epibrassinolide were more thermotolerant than untreated plants (Dhaubhadel et al., 1999; Dhaubhadel et al., 2002; Wilen et al., 1995). The improved thermotolerance of treated seedlings was due to increased translation of HSP mRNAs and greater stability of the translational machinery, but a mechanism for these improvements has not been determined. It is uncertain if the improved HSP translation and stability of the translational apparatus induced by epibrassinolide are related. Although epibrassinolide improves HSP translation, no increase in HSP transcription was observed (Dhaubhadel *et al.*, 2002).

1.7.1 Small Molecular Weight HSPs

Small HSPs range in size from 16-30 kD (Helm et al., 1997; Waters et al., 1996) and are the most abundantly produced HSPs in plants during heat stress (Vierling, 1990; Waters et al., 1996). sHSPs are thought to function by preventing or slowing the denaturation of proteins. Their synthesis has been observed in heat treated pea leaves, seeds and flowers (Hernandez and Vierling, 1993), soybean roots (Jinn et al., 1997) and *Arabidopsis* seedlings (Takahashi and Komeda, 1989). Developing microspores also produce a high amount of sHSP mRNAs, peaking early in development and decreasing

towards maturity (Atkinson et al., 1993; Mascarenhas and Crone, 1996). Chloroplasts also accumulate sHSP during heat stress (Süss and Yordanov, 1986) as do *Arabidopsis* mitochondria (Visiolo et al., 1997). sHSCs have been shown to be constitutively expressed in maize tissues (Cooper et al., 1984).

In six different legume species, at least 5 different sHSPs were detected during heat stress (Hernandez and Vierling, 1993). The *HSP18.1* genes were expressed in flowers at tissue temperatures approximately 5°C lower than required for their induction in leaf tissue. In this instance, whole flowers were used and the high level of *HSP18.1* expression may have been a result of developmental control (i.e. HSC rather than HSPs) as well as heat shock induction. *HSP18.1* is thought to be developmentally expressed to protect proteins in desiccating tissues, such as seeds and pollen. As only an antibody to pea HSP18.1 was used to detect these proteins, the expression patterns of the other sHSP families were not determined.

The HSP18.1 and HSP17.1 proteins from pea form homododecamers to prevent protein aggregation *in vitro* (Lee et al., 1995a). Homododecamers of these sHSPs increased the renaturation of chemically denatured citrate synthase and lactate dehydrogenase in a manner independent of ATP. Aggregation and precipitation of citrate synthase and lactate dehydrogenase was prevented at 45°C in the presence of HSP18.1 or HSP17.1. Heat treating these enzymes at 38°C resulted in protein denaturation; however, up to 60% of enzyme activity was restored if either HSP18.1 or HSP17.1 were present in the mixture. These results clearly show that, *in vitro*, HSP18.1 and HSP17.1 can protect proteins during heat stress (Lee et al., 1995a).

Heat denaturation of transgenic luciferase was found to be slower in heat shocked *Arabidopsis* cell suspension cultures over-expressing *AtHSP17.6* (Forreiter et al., 1997). While the over-expression of *AtHSP17.6* slowed protein denaturation the sHSP was unable to totally prevent denaturation. The authors suggested that sHSPs slowed protein denaturation and prevented irreversible protein denaturation. Irreversibly denatured proteins would be targeted for degradation whereas reversibly denatured proteins could be refolded by other HSPs after removal of the HTS. Some proteins, protected by sHSPs during heat shock, would be able to spontaneously refold without

assistance from a chaperonin. The rate of refolding and the amount of protein restored are likely to be lower in the absence of chaperonins, however.

The expression of sHSCs in seeds and pollen is developmentally controlled (Atkinson et al., 1993; Smykal et al., 2000; Waters et al., 1996). These sHSCs are thought to prevent irreversible protein denaturation during desiccation (Waters et al., 1996).

1.7.2 HSP60 Class

The ATP-dependent HSP60 chaperonins form tetradecamers of α and β subunits in procaryotes, mitochondria and plastids (Nover and Scharf, 1997). Chaperonin 60 is the major chaperonin found in chloroplasts and is orthologous to the bacterial chaperonin GroEL (Schmitz et al., 1996). Chaperonin 60 was first described as a RuBisCo binding protein due to its function in the assembly of this enzyme. Both α and β subunits of Chaperonin 60 are constitutively expressed in chloroplasts and are not heat-induced. The levels of β subunit mRNA and protein are regulated by light and leaf development, however.

1.7.3 HSP70 Class

In *Arabidopsis*, the HSP70 class of proteins has diverse functions with members synthesized constitutively as well as under stress conditions (Sung et al., 2001). In spinach, expression patterns of twelve HSP70s were identified in various cellular components, including chloroplasts, mitochondria and endoplasmic reticulum (Li et al., 1999). All of the ten HSP70s examined were expressed in leaves after one hour at heat stress conditions (37°C). Maintenance of expression over longer periods was observed only for some of the genes, suggesting that members of this family have different roles in the cell. In *Arabidopsis* (Sung et al., 2001), soybean (Roberts and Key, 1991) and pea (DeRocher and Vierling, 1995) several different HSP70 members, with different expression patterns, were identified.

HSP70 proteins are thought to be chaperonins. *S. cereviseae* SSA1, a HSP70 class protein, binds to other proteins *in vitro* to prevent their irreversible aggregation (Glover and Lindquist, 1998). SSA1, HSP40 and HSP104 are thought to form a

heterotrimer that facilitates protein disaggregation and renaturation after chemical or heat denaturation (Glover and Lindquist, 1998).

Petunia HSP70 alone did not restore transgenic luciferase activity in heat stressed *Arabidopsis* cell suspension cultures (Forreiter et al., 1997). The expression of petunia HSP70 together with AtHSP17.6 was able to restore 90% of the luciferase activity, however. sHSPs are thought to prevent irreversible protein denaturation thereby preserving proteins in a form that can be refolded by HSP70 and/or other chaperonins. Without sHSPs, proteins become irreversibly denatured and cannot be refolded by HSP70.

Transgenic *A. thaliana* plants containing an antisense *HSP70* had lower levels of both HSC70 and heat-induced HSP70 as well as a lower thermotolerance than wild-type plants (Lee and Schöffl, 1996). In *S. cereviseae* a HSP70 class protein, LHS1P, refolds proteins in the endoplasmic reticulum denatured by heat (Saris et al., 1997). From the experiments discussed here it appears that heat-induced HSP70s play a role in refolding denatured proteins after heat shock.

Another important role for HSP70s is the regulation of heat shock transcription factor activity (Lee and Schöffl, 1996. See "Control of HSP expression" below; Prändl et al., 1998).

1.7.4 HSP90 Class

HSP90 is constitutively expressed and can comprise almost 1% of the total protein of plant cells (for recent review see Buchner, 1999). *HSP90* is necessary for thermotolerance as demonstrated in the thermosensitive *Arabidopsis tu8* mutant, which cannot produce cytosolic HSP90 (Ludwig Muller et al., 2000). HSP90 also chaperones protein folding but has a limited range of protein substrates compared to other chaperonins. Steroid hormone receptors and kinases are amongst HSP90 substrates (Buchner, 1999). HSP90s may also be involved in the regulation of heat shock transcription factor activity (Buchner, 1999).

HSP90 is strongly upregulated by HTS, low temperature stress (Krishna et al., 1995; Ludwig Muller et al., 2000; Marrs et al., 1993; Queitsch et al., 2002) and other stresses causing protein denaturation (Kampinga et al., 1995). A shortage of available

HSP90 in the cells, resulting from a HTS or application of the antibiotic geldanamycin (which specifically targets HSP90 (Buchner, 1999)), had epigenetic effects on the phenotype of recombinant, inbred *Arabidopsis* lines (Queitsch et al., 2002).

1.7.5 HSP100 Class

HSP101 in plants and the orthologous *S. cereviseae* and bacterial genes, *HSP104* and *ClpB* respectively, are necessary for organismal thermotolerance (for recent reviews of plant HSP101 see Agarwal et al., 2002; Katiyar-Agarwal et al., 2001). Mutating or deactivating *HSP101* or its orthologues decreased thermotolerance (Hong et al., 2000; Hong et al., 2001; Parsell et al., 1994). An indication of the high degree of conservation in the *HSP101/HSP104* family was the ability of plant (*Arabidopsis*, soybean, wheat and maize) *HSP101* to complement *S. cereviseae hsp104* mutants (Lee et al., 1994; Schirmer et al., 1994; Young et al., 2001). Although thermotolerance was not completely restored in these yeast cells, there was a significant improvement in *hsp104* mutant cells complemented with the plant *HSP101* (Lee et al., 1994; Schirmer et al., 1994). Activity of a transgenic luciferase in heat shocked *hsp104* and wild-type *S. cereviseae* was 5% of that in cells grown at normal temperatures. When allowed to recover from the heat shock, luciferase activity was not restored in the *hsp104* mutants compared to a 90% recovery in wild-type strains (Parsell et al., 1994). Analysis of cell lysates, after recovery from the heat stress, showed that of *hsp104* mutant cells contained aggregates of denatured proteins, formed during the heat shock, unlike wild-type cells which resolubilised them.

HSP101 acts by refolding denatured proteins after a HTS. It has two ATP-binding domains per molecule and forms dodecamers *in vivo* around the denatured protein (Glover and Lindquist, 1998; Parsell et al., 1994).

In *S. cereviseae*, HSP104 chaperone activity *in vitro* requires the presence of two other HSP-related proteins, *YDJ1* (HSP40 Class) and *SSA1* (HSP70 Class) (Glover and Lindquist, 1998). When luciferase was chemically denatured and mixed with cell lysates containing all three HSP proteins present, 50-70% of enzyme activity was restored (Glover and Lindquist, 1998). Recovery was only 10-15% when cell free lysates from mutant strains lacking any one of these three HSPs was used (Glover and

Lindquist, 1998). YDJ1 and SSA1 limited the aggregation of denatured proteins but each alone or both together did not enable protein refolding after the denaturating agent was removed. HSP104 by itself did not prevent or limit aggregation of denatured proteins, nor could it refold them after removal of the denaturating agent. If all three HSPs were present, aggregation of denatured proteins was prevented and refolding was observed after removal of the denaturating agent (Glover and Lindquist, 1998). How YDJ1 and SSA1 assemble around the HSP104 dodecamer is not known. Assembly and function of the polyprotein complex is highly conserved however, as YDJ1 can be replaced with its bacterial orthologue DnaJ (Glover and Lindquist, 1998).

It is not known if plant HSP101 requires proteins orthologous to YDJ1 (HSP70) and SSA1 (HSP40) for protein renaturation but proteins with relatively high levels of amino acid similarity to YDJ1 and SSA1 (60% and 83%) have been identified in plants (personal observations).

HSP101 is expressed in developing *Arabidopsis* seeds (Hong et al., 2001; Queitsch et al., 2000) and *Z. mays* pollen (Young et al., 2001). It is also strongly expressed in HTS-treated tissues (Hong et al., 2000; Queitsch et al., 2000). In non-HTS-treated maize, *HSP101* mRNA and protein was found in most tissues, except mature pollen. The amount of *Z. mays HSP101* mRNA and protein increased in all tissues when HTS-treated except in mature pollen (Young et al., 2001).

There are at least three *HSP101* isoforms in *Arabidopsis*, At1g74310, At4g14670 and At5g57710. Mutations in the At1g74310 *HSP101* gene, such as the *hot1 Arabidopsis* mutants (Hong et al., 2000; Hong et al., 2001) or plants with otherwise reduced HSP101 protein expression (Queitsch et al., 2000), had lower thermotolerance than wild-type plants (Hong et al., 2000; Hong et al., 2001; Queitsch et al., 2000). The function and expression patterns of the other two *HSP101* isoforms have not been determined. Based on the observation of decreased thermotolerance in the *hot1* mutants it appears that the two isoforms cannot compensate for mutations in At1g74310. Whether this is because the two isoforms cannot be transcribed and translated in seedling tissues or because of functional differences between the isoforms is not known.

1.7.6 Control of HSP expression

HSP expression is under the control of heat shock factors (HSFs), sometimes called heat shock transcription factors (Schöffl et al., 1998). These transcription factors bind to conserved sequences, heat shock elements (HSEs), found in the promoters of most, if not all, heat inducible HSPs (Rieping and Schöffl, 1992). HSFs have been cloned from several plant species including tomato, soybean, maize, *A. thaliana* and *B. napus* (Nover and Scharf, 1997). At least four HSFs have been identified in *Arabidopsis* and six in soybean (Czarnecka-Verner et al., 1995). *GmHSF5* and *GmHSF31* are constitutively expressed in soybean leaves; transcription of all six soybean HSFs increases during heat stress. In *Arabidopsis*, *HSF3* and *HSF4* are both constitutively expressed, but only *HSF4* expression increases in heat stressed leaves (Prändl et al., 1998).

Under non-heat shock conditions, HSFs form heterodimers with HSP70. During heat shock the heterodimers disassociate and HSF forms a homotrimer (Prändl et al., 1998) which binds to the various HSEs in HSP promoters, thereby activating transcription of HSP genes (Prändl et al., 1998). In heat shocked tobacco cell lysates, HSF binding to HSEs was reversibly inhibited by ATP (Shimizu et al., 1996), possibly limiting activation of the heat shock response until the cell is under stress, i.e., ATP limited.

The HSP70/HSF complex is highly dependent on HSF conformation. A transgenic HSF1-GUS fusion protein expressed in *A. thaliana* was not bound by HSP70 and formed homotrimers with itself and heterotrimers with the endogenous HSFs at non-heat shock temperatures (Lee et al., 1995b). Trimers containing a HSF1-GUS monomer were able to induce HSP expression under non-stress conditions. These observations indicate that HSF binding to HSP70 is specific and disruption of this relationship results in a loss of post-translational regulation of the transcription factor. The specificity of the HSF-HSP70 interaction is evidenced by the expression of *Arabidopsis* HSF3 in tobacco. The AtHSF3 was not bound by endogenous tobacco HSP70; instead mixed trimers of both tobacco and *Arabidopsis* HSFs were formed (Prändl et al., 1998). These trimers were able to bind to HSEs and induce HSP expression at non-inductive temperatures.

The regulation of HSP expression by other factors is poorly understood. The mechanism by which epibrassinolide increases *HSP* mRNA translation is not known (Dhaubhadel et al., 2002) nor is the mechanism by which cross-tolerance induces increased HSP synthesis. Post-transcriptional regulation of HSP protein synthesis is evident since accumulation of *HSP* mRNAs does not always coincide with increases in HSP production (Young *et al*., 2001; Dhaubhadel *et al.*, 2002).

1.8 THE FLORAL DEVELOPMENT GENE *LEAFY*

LEAFY (LFY) is a transcription factor that is important in the regulation of floral development. It has two roles: the initiation of a floral meristem from an inflorescence meristem (Weigel et al., 1992; Weigel and Meyerowitz, 1993) and control of flower organ development (Busch et al., 1999). In *Arabidopsis lfy* mutants, the first few flowers on the inflorescence partially develop into inflorescences (Yanofsky, 1995). Flowers developing higher up the inflorescence develop into flowers but the individual organs have a leaf-like morphology. LFY initiates transcription of the floral organ-identity genes *AGAMOUS*, *APETALA* and *PISTILLATA* (Yanofsky, 1995, Busch et al., 1999).

CHAPTER 2 HIGH TEMPERATURE STRESS DURING JULY NEGATIVELY AFFECTS YIELDS OF BARLEY, CANOLA, FLAX, DURUM AND SPRING WHEAT IN SASKATCHEWAN

2.1 INTRODUCTION

Abiotic stresses adversely affect crops world wide causing yields to fall below their maximum genetic potential (Boyer, 1982). In Saskatchewan, with its semi-arid agricultural conditions, drought and high temperature (HT) stress are presumed to be the major abiotic stressors. Drought and HT stress have been shown to adversely affect a wide variety of crops to differing degrees (Begg and Turner, 1976; Boyer, 1996; Chipanshi et al., 1999; McCaig, 1997; Nuttal et al., 1992). It is not known if each stressor affects yields in different crops at the same developmental stage and to the same degree. That is, do HT stress and/or drought stress act to reduce yields in all crops by inhibiting growth at the early vegetative, late vegetative, flowering or grain filling stage(s) of development? Correlations of reduced yields with the onset of stress inducing conditions in different species might suggest that a stressor affected different plant species in a similar manner. Such correlations in different crop species would be of interest to plant breeders, crop physiologists and producers.

Drought and HT stress adversely affect yields in different ways. Drought stress may result in reduced photosynthetic activity, changes in morphology (especially root development) and reduction or change in harvest index (reviewed in Begg and Turner, 1976; Boyer, 1996; Maiti et al., 2000). Drought stress at any stage of development, especially during flowering, caused reductions in *Brassica napus* L. (canola) yields (Hashem et al., 1998). In wheat, reductions in yield were observed when drought stress occurred preanthesis (Entz and Fowler, 1990). Changes in harvest index in response to drought stress indicate an altered distribution of resources within a plant,

which in turn affects seed development (Entz and Fowler, 1990; Hashem et al., 1998; Maiti et al., 2000).

HT stress can also have an effect on many stages of plant development (e.g. as reported in wheat, Porter and Gawith, 1999) but the greatest detrimental effects of HT stress occur during flowering. High temperature stress of this stage reduced yields of canola (Chapter 3, Angadi et al., 2000; Nuttal et al., 1992), *Triticum aestivum* L. (wheat, Ferris et al., 1998; McCaig, 1997; Porter and Gawith, 1999) and *Linum usitatissimum* L. (flax, Cross et al., 2003; Dybing and Zimmerman, 1965; Gusta et al., 1997). Functions of the reproductive structures (as opposed to the vegetative structures) were reported to be the most affected by HT stress in several plant species (Chapter 3, Cross et al., 2003; Polowick and Sawhney, 1987; Saini et al., 1983; Sato et al., 2002). Consequently, increased temperatures during flowering resulted in decreased seed production.

I wished to determine if HT stress or its associated drought stress in the field had the greater effect on crop yields. Furthermore, I wanted to determine if different crops were affected at the same developmental stage, thereby suggesting that the stressor(s) induced a common response across a range of species. Statistical analyses of *Hordeum vulgare* L. (barley), canola, flax, *T. durum* Desf. (durum wheat) and spring wheat yields from five locations across Saskatchewan over a 25-year time period (1976-2000) identified the contribution of both drought and HT stress to reduced crop yields. Each stressor affected a different stage of crop development but only high July temperatures (corresponding with flowering in these species) correlated with a reduction in yields in all five crop species.

2.2 Materials and Methods

2.2.1 Temperature, precipitation and crop yield data.

Meteorological data, total precipitation (mm) and mean maximum daily temperatures (MMDT; °C) for the months of May-Sept., from 1976-2000, were obtained from Environment Canada for the Melfort (predominantly Dark Gray and Black Chernozemic soil types; 52°49'N 104°36'W), Saskatoon (Dark Brown Chernozemic; 52°10'N 106°43'W), Scott (Dark Brown and Gray Chernozemic; 52°22'N 108°50'W), Wynyard (Black Chernozemic; 52°46'N 104°12'W) and Yorkton (Black Chernozemic;

52°13'N 102°28'W) weather stations. Each of these areas was considered an independent, random variable for "Location". Total precipitation was selected as indicative of cumulative precipitation. MMDT was selected as peak temperature because it gives a better indication of the maximum level of stress than does mean temperature, which indicates average accumulated "degree.time" units. Total precipitation from the winter preceding each growing year (Oct. to Apr.) was also used in the analysis.

Yield data (bu collected at the elevator per acre harvested), for the years 1976-2000, for barley, canola (both *B. napus* and *B. rapa* combined), flax, spring and durum wheat were obtained for the five or six Rural Municipalities (RM) surrounding each of the five urban locations mentioned above (Saskatchewan Agriculture, Food and Rural Revitalization website; www.agr.gov.sk.ca). The mean crop yields from the five or six RMs were used in the analyses. Data for three years of durum yield at Melfort were missing from the database.

2.2.2 Statistical treatment of data

Statistical analyses were performed using the SPSS statistical software package (SPSS Inc, Chicago, IL). First a multivariate analysis of variance (MANOVA) procedure was performed, using all crop yields as dependant variables; all other variables (May-Sept. MMDT, May-Sept. precipitation, total winter precipitation, year and location) were incorporated into the model as independent variables (cofactors in SPSS). The results using Pillai's Trace are given and are identical to output using Wilk's Lambda, Hotelling's Trace and Roy's Largest Root, except for the location variable (which still had a significant affect). A breakdown of the effect of each independent variable on each crop, from the MANOVA, enabled the identification of variables with a significant effect on crop yield, for use. Variables identified as having a significant effect of the yield of a crop were used in a univariate analysis of variance.

Meteorological variables with a significant effect on an individual crop were subsequently used in a univariate analysis of variance (UNIANOVA). One UNIANOVA was performed for each crop using each independent variable as a covariate (in the SPSS Univariate GLM procedure). If location was determined to be

significant for a crop via the MANOVA, then location was used as a random factor (in the GLM procedure), forcing the analysis to be performed separately for each location. Only those variables determined to be significant by the multivariate analysis were included; however, the results was similar when all data was included in the univariate analysis (replicating the results from the MANOVA). If location was not deemed significant from the MANOVA (as was the case for flax, barley and canola), then the data from the different locations were pooled to give a larger data set.

Variables with a significant effect on yield of each crop (from the UNIANOVA) were used in multiple regressions to determine the contribution of each variable. Significant variables from the UNIANOVA were used as independent variables, with crop yields as dependant variables in the regression analysis. The location factor was not included in the regression analysis for wheat and durum models, (where location was determined to have a significant affect) as this was a non-numeric variable.

2.3 RESULTS

The MANOVA identified several variables with significant correlations with crop yields (Table 2.1). At least two variables were found to have a significant effect on crop yield for each of the five crops. The overall R-squared/Adjusted R-squared values for the wheat, durum, flax, barley and canola models in the MANOVA were 0.651/0.597, 0.655/0.603, 0.290/0.181, 0.295/0.181 and 0.551/0.482 respectively. Location did not significantly affect yields of flax, barley or canola.

Both precipitation and temperature had significant effects on crop yields, as determined by UNIANOVA (Table 2.2 and summarized in Table 2.3). High temperature significantly affected yields of all crops in July. June MMDT was a significant factor in determining yields of all crops except barley. High temperatures throughout the summer (June-Sept.) were important in determining yields of both spring and durum wheat (see below).

Monthly precipitation did not have a large role in determining yields, except in early spring (Tables 2.2 and 2.3). High levels of precipitation during May had positive effects on yields of spring and durum wheat and canola, while total precipitation from the preceding winter also had a positive effect on wheat and barley yields (Table 2.4).

Table 2.1 Monthly precipitation and MMDT variables with significant correlation to all crop yields.

F values from the MANOVA are shown below, using Pillai's Trace. Variables with a significant effect on all crop yields (at $p \leq 0.05$) are indicated with an asterisk. Hypothesis and Error degrees of freedom for all variables, except Location, were 5 and 101 respectively. Location had 20 and 416 degrees of freedom.

Meteorological variable	F	p
May MMDT	2.438	0.039*
June MMDT	6.810	0.000*
July MMDT	14.181	0.000*
Aug. MMDT	3.069	0.013*
Sept. MMDT	2.473	0.037*
May Ppt.	2.835	0.019*
June Ppt.	2.326	0.048*
July Ppt.	1.547	0.182
Aug. Ppt.	0.746	0.591
Sept. Ppt.	1.030	0.404
Winter Ppt.	4.796	0.001*
Location	2.018	0.006*
Year	4.593	0.001*

Table 2.2 UNIANOVA of monthly precipitation and MMDT on individual crops.

A UNIANOVA was performed to determine the variables, from the MANOVA, that significantly affected yield. A separate UNIANOVA was performed for each crop. For flax, barley and canola, location was not significant (as determined in the MANOVA) and the data from each of the five locations were pooled for analysis.

Crop	Independent Variable	F	p
Wheat	June MMDT	24.085	0.000
	July MMDT	49.472	0.000
	Aug. MMDT	18.126	0.000
	Sept. MMDT	8.280	0.005
	May Ppt.	20.609	0.000
	June Ppt.	1.256	0.265
	Winter Ppt.	12.321	0.001
	Location	3.859	0.006
Durum	June MMDT	47.525	0.000
	July MMDT	42.850	0.000
	Aug. MMDT	21.664	0.000
	Sept. MMDT	20.500	0.000
	May Ppt.	12.187	0.001
	Location	3.746	0.007
	Year	3.834	0.053
Flax	June MMDT	6.083	0.015
	July MMDT	10.561	0.001
Barley	July MMDT	17.926	0.000
	Winter Ppt.	9.343	0.003
Canola	May MMDT	0.586	0.446
	June MMDT	4.268	0.041
	July MMDT	59.202	0.000
	May Ppt.	5.822	0.017

Table 2.3 Summary of p values from the UNIANOVA for variables with a significant effect on crop yields.

Numbers are p values for UNIANOVAs performed on each crop for the specified variable. Variables with significant effects on crop yields (at $p \leq 0.05$) are indicated with an asterisk. Dashes represent variables deemed not significant in the multivariate analysis of variance and therefore not used in the analysis. R-squared and Adjusted R-Squared values are shown for those models where location was not a factor in the analysis.

Variable	Wheat	Durum	Flax	Barley	Canola
May MMDT	-	-	-	-	0.446
June MMDT	0.000*	0.000*	0.017*	-	0.041*
July MMDT	0.000*	0.000*	0.001*	0.000*	0.000*
Aug. MMDT	0.000*	0.000*	-	-	-
Sept. MMDT	0.005*	0.000*	-	-	-
May Ppt.	0.000*	0.000*	0.010*	0.400	0.017*
June Ppt.	0.265	-	-	-	-
July Ppt.	-	-	-	-	-
Aug. Ppt.	-	-	-	-	-
Sept. Ppt.	-	-	-	-	-
Tot. Wint. Ppt.	0.006*	-	-	0.003*	-
Location	0.006*	0.007*	-	-	-
Year	-	0.053	-	-	-
R-Squared			0.206	0.196	0.418
Adj. R-Squared			0.186	0.176	0.398

Table 2.4 Regression coefficients of regression equations for models.

Regression analyses used the variables with significant effects on crop yield (determined via UNIANOVA) as independent variables and crop yield as the dependent variable. The regression coefficient (slope) of the regression equations for these analyses are shown. All the independent variables were significantly correlated with the dependant variable (standard error and p values given). For spring wheat and durum wheat, only the significant numerical values from the UNIANOVA were included in the regression (i.e. Location was not used).

Crop	Independent variable	Regression coefficient (Slope)	Std. Error	p	Correlation Coefficient (R)
Wheat	June MMDT	-1.434	0.211	0.000	0.744
	July MMDT	-1.980	0.264	0.000	
	Aug. MMDT	0.905	0.175	0.000	
	Sept. MMDT	0.821	0.172	0.005	
	May Ppt.	0.041	0.013	0.000	
	Winter Ppt.	0.032	0.011	0.005	
Durum	June MMDT	-1.434	0.218	0.000	0.769
	July MMDT	-1.980	0.274	0.000	
	Aug. MMDT	0.905	0.180	0.000	
	Sept. MMDT	0.821	0.176	0.000	
	May Ppt.	0.041	0.013	0.003	
Flax	June MMDT	-1.347	0.559	0.017	0.454
	July MMDT	-2.196	0.664	0.001	
	May Ppt.	0.087	0.033	0.010	
Barley	July MMDT	-2.871	0.678	0.000	0.438
	Winter Ppt.	0.100	0.033	0.003	
Canola	June MMDT	-0.325	0.167	0.054	0.644
	July MMDT	-1.530	0.198	0.000	
	May Ppt.	0.024	0.010	0.019	

Based on the initial analysis, we hypothesized that May and/or winter precipitation would have a positive effect on flax and barley yields (indicated in Table 2.3). Accordingly, UNIANOVAs including these two variables were performed again for these two crops, showing that May precipitation had a significant effect on flax yields, but not barley. Furthermore, a MANOVA using pooled values for summer precipitation (May-Aug.) revealed that total summer precipitation did not have a significant effect on crop yields (data not shown).

Location had a significant effect on spring wheat and durum yields, showing a high degree of variation in yield, depending on location. Crop yields did not vary with year, suggesting that, over the 25-year time period, yields have not improved. The possible exception is durum wheat, where $p = 0.053$ (Table 2.2). One possible reason for the near-significance of year on durum yields was an increase in the number of producers sowing the grain near Melfort. A reanalysis of the data, neglecting the Melfort variables, revealed that year was not a significant variable on durum yields in the four other locations (data not shown).

The R-squared / Adjusted R-Squared values for the flax, barley and canola models are shown in Table 2.3. The low values for flax and barley suggest that the models do not fit the data very well and the regression would probably not be considered significant. For canola, the R-squared/Adjusted R-squared values were 0.418 and 0.398, respectively.

July and June MMDT were highly negatively correlated with yield, as indicated by the significant regression coefficients for each crop (Table 2.4, regression coefficients), with July MMDT having the largest effect of all the variables. In durum and spring wheat, June and July MMDT were negatively correlated with yield, whereas Aug. and Sept. MMDT were positively correlated with yield. May and/or total winter precipitation had positive effects on the yields of all the crops analysed.

2.4 Discussion

My analysis, based on widely separated areas of Saskatchewan, supports previous reports (at the plot and farm size level) which found that HT stress of canola (Nuttal et al., 1992) and wheat (McCaig, 1997) during July (flowering) have a

detrimental effect on seed production. Other studies of the effects of HT stress on wheat also support these findings (Chipanshi et al., 1999; Entz and Fowler, 1990; Ferris et al., 1998; Porter and Gawith, 1999).

HT stress experiments in growth chambers using flax (Cross et al., 2003; Dybing and Zimmerman, 1965; Gusta et al., 1997), canola (Chapter 3, Angadi et al., 2000; Hashem et al., 1998; Polowick and Sawhney, 1988) and wheat (Saini et al., 1983) have demonstrated that gametophyte development and/or function is adversely affected in these species. My field data analyses support these "in house" experiments. HT stress during flowering causes reductions in yields in a variety of crop species.

HT stress during June and July, when the plants were flowering, caused a reduction in spring wheat, durum wheat, flax, barley and canola yields. All of the crops in this study were affected by high July temperatures, suggesting that HT stress caused reductions in seed set via a common mechanism and/or that the same, thermosensitive, developmental stage is reached during July (and late June for all crops except barley) in the different species. Except for barley, the effect of June MMDT on crop yield was less significant than July MMDT (Tables 2.3 and 2.4). If seeded in early spring, all of these crops begin flowering in the latter half of June.

A positive correlation between August and September MMDT and yield was observed for spring and durum wheat (Table 2.4) suggesting that accumulation of seed reserved is enhanced by high temperatures. My analyses support previous observations that wheat becomes more thermotolerant as the plants mature (Porter and Gawith, 1999). Temperatures in months other than June and July did not have a significant effect on flax, barley or canola yields, suggesting that vegetative growth and seed development/maturity are not negatively affected by HT stress in these crops.

The observation that drought stress did not have a significant effect on yields, other than in spring, was not expected, given that precipitation at times other than in spring is essential for plant growth and development. That years with below average precipitation did not negatively affect yields may be due to the way plants respond to and/or tolerate drought stress compared to HT stress. Higher winter and spring precipitation had a positive effect on crop yields suggesting that early establishment of seedlings improved subsequent stages of development. Poor seedling establishment

could delay subsequent stages of development and/or reduce total vegetative biomass (Begg and Turner, 1976).

Drought stress can indirectly affect crop yields, through limiting carbohydrate synthesis and accumulation thereby reducing the ability of the plant to support seed development (Begg and Turner, 1976; Boyer, 1996; Maiti et al., 2000). Seed and/or fruit development delayed by drought stress may return to normal once a drought stress is relieved. In comparison, HT stress has a direct effect on seed production, resulting in dysfunction of the gametophytes (Chapter 3, Polowick and Sawhney, 1988; Saini et al., 1983). Flowers relieved of HT stress are unlikely to be able to produce seeds and/or fruit as damage to the gametophytes would probably already have occurred. Production of seeds by plants relieved from a HTS would more likely be from flowers that developed after the HT period.

A number of assumptions were made in this analysis. It was assumed that all of the harvested crop was deposited (and thus recorded) at the grain elevator. Seed retained for the following year's crop or stored on the farm was not included in the yield data. Furthermore, during high stress years, it may be more economical to use crops for hay or silage, rather than harvest them. Crop yields reported in high stress years may have underestimated the actual amount of seed produced, therefore. Another source of error is the lack of distinction between fall and spring planted crops, as fall planted crops would have a better chance of avoiding HT stress. The relative contribution of fall planted crops is small, however. For example, winter wheat in Saskatchewan is seeded on less than 100,000 ha annually compared to spring wheat, which is planted on 5 – 7 million ha yearly (Saskatchewan Agriculture, Food and Rural Revitalization).

Meteorological data were collected at a single location to represent weather patterns over a geographical area. While air temperatures would not be expected to vary much over the area, precipitation could have varied significantly. In addition, precipitation and MMDT were found to be highly co-linear within months. For example, June and July MMDT and precipitation were significantly correlated at the 1% level, with correlation coefficients (R) of –0.339 and –0.549, respectively, making it difficult to distinguish between the effect on yield of each variable separately. When

July MMDT was excluded from the MANOVA, July precipitation was not significant, suggesting that MMDT and precipitation are independent of each other.

The models used did not accommodate non-linear crop responses to HT and/or drought stress. Two possible non-linear responses could be an exponential decline in yields with increasing severity of stress or a reduction in yield starting only above a threshold stress level. The low R-squared values for the flax and barley models may have been due to a non-linear response to HT and/or drought stress, or simply because unidentified factors have a significant role in determining the yields of these crops. Previous work has reported in flax that HT stress during flowering reduced seed production, however (Cross et al., 2003; Dybing and Zimmerman, 1965; Gusta et al., 1997). Another difficulty inherent in the data is that many variables are not normally distributed. For example, in the case of all yield data except barley, the precipitation data and June MMDT are significantly skewed. A logarithmic transformation of the data did not reveal any major differences in the results (not shown).

2.5 Conclusions

My analysis suggests that HT stress in the field, especially during flowering, was the major stressor affecting crop yields, for spring wheat, durum wheat and canola. Flax and barley were also affected by HT stress during July, but not to the same degree as wheat and canola. Water availability in early spring was an important factor for seedling establishment and early growth and thus had consequences on later developmental stages, including seed production. In Saskatchewan, drought and HT stress are correlated to a high degree, both having adverse effects on crop yields.

CHAPTER 3 HIGH TEMPERATURE STRESS OF BRASSICA NAPUS DURING FLOWERING REDUCES BOTH MICRO- AND MEGA-GAMETOPHYTE FERTILITY AND DISRUPTS EMBRYO DEVELOPMENT

3.1 INTRODUCTION

The adverse effects of high temperature stress (HTS) on plant reproduction have implications in crop production systems and impact on the geographical distribution of plant species. Understanding the effects of HTS on gametophyte development and possible mechanisms to overcome HTS-induced inhibition of seed production are important in order to improve crop production under HTS conditions.

Both monocotyledonous (Carlson, 1990; Saini et al., 1983) and dicotyledonous (Angadi et al., 2000; Cross et al., 2003; Morrison, 1993; Nuttal et al., 1992; Peet et al., 1998) plants experiencing HTS during flowering suffer a reduced seed set. The range of species adversely affected by HTS during the reproductive stage suggests that some common mechanisms are involved in this HTS-induced reduced seed production.

Previous reports in *Brassica napus* (L) suggest that reduced seed set during HTS was due to reduced gametophyte fertility or function (Angadi et al., 2000; Morrison, 1993; Polowick and Sawhney, 1988). Neither the stage of flowering most sensitive to HTS nor the gametophyte most affected by HTS has been identified. Reduced pollen viability due to HTS has been reported in *Phaseolus vulgaris* (L) (48 hours at 35°or 41°:21°C light:dark Weaver et al., 1985), *Lycopersicon esculentum* (Mill.) (32°:26°C light:dark for various durations Peet et al., 1998; Sato et al., 2002), *Triticum aestivum* (L) (30°:20°C for 72 hours Saini and Aspinall, 1982) and *Zea mays* (38°/32°C for 24 or 48 hours: Herrero and Johnson, 1980) while abnormal megagametophyte development has

been reported in *B. napus* (Polowick and Sawhney, 1988), *L. esculentum* (Sato et al., 2002) and *T. aestivum* (Saini et al., 1983).

Improved thermotolerance in plants has resulted from the synthesis of isoprene (Singsaas et al., 1997) or glycine betaine (Sakamoto and Murata, 2001), the production of antioxidant enzymes such as dehydroascorbate reductase (Kubo et al., 1999) and reductions in □-linolenic acid concentrations (Murakami et al., 2000). Not all plants have the genetic makeup that enables them to use these thermotolerance mechanisms, however. One universal means of improving thermotolerance is the production of Heat Shock Proteins (HSPs: Nagao et al., 1986). Both small HSPs (sHSPs Malik et al., 1999; Park and Bong, 2002) and HSP101 (Hong et al., 2000; Hong et al., 2001; Queitsch et al., 2000) are required for improved resistance to heat shock in plant cells, leaves or seedlings. Knowledge of HSP production in the gametophytes is incomplete, however. *sHSPs*, *HSP70*, *HSP81, HSP82, HSP90* and *HSP101* are transcribed in a similar pattern in the microspores of various species (Atkinson et al., 1993; Duck and Folk, 1994; Dupuis and Dumas, 1990; Marrs et al., 1993; Smykal and Pechan, 2000 2001). That is, coincident with microspore meiosis there is a high level of *HSP* transcription which declines as the microspores mature into pollen. Developing microspores respond to HTS by increasing *HSP* transcription; however, the ability to respond to HTS drops to negligible levels as the microspores mature. Determining HSP protein synthesis in HTS-treated mature pollen grains is difficult as increases in *HSP* transcription are not necessarily correlated with increased HSP protein synthesis (Dhaubhadel et al., 2002; Young et al., 2001) and mature pollen grains do not take up ^{35}S-methionine.

Data on the production of HSPs in germinating pollen are contradictory. *HSP* mRNA was observed in germinating lily and tobacco pollen (Herpen et al., 1989) but not in *Tradescantia paludosa* pollen (reviewed in Mascarenhas and Crone, 1996). In contrast to the situation in microgametophytes, the female reproductive tissues appear to be able to mount a heat shock response. *Z. mays* silks were able to synthesize a full set of HSPs before maturity (Dupuis and Dumas, 1990). *HSP101* mRNA and protein has also been observed in silks developing under non-HTS conditions (Young et al., 2001).

It is not known if HSP synthesis occurs in *B. napus* mature pollen or female gametophytes and which HSPs, if any, are synthesized during HTS. I used RT-PCR to

examine the amount of three HSP mRNAs present in both HTS and control pollen, pistils and leaves with the aim of understanding the role of HSPs in gametophyte thermotolerance.

In this chapter I report the effects of HTS on flowering in *B. napus*, in particular the effects of HTS on microgametophyte development as well as HSP mRNA production in pollen and pistils developing under HTS and control conditions. *B. napus* (canola) is an important crop in Western Canada that suffers from high yield losses during hot summers, a fact that makes it important to understand the physiology of HTS-induced reductions in seed set. Other plants are also adversely affected by HTS during reproduction (see Chapter 2), so that an understanding of the effects of HTS on the reproductive tissues and seed set in *B. napus* should provide insights into the effects of HTS in other species.

3.2 Materials and Methods

3.2.1 Plant growth and HTS treatment

The doubled haploid *B. napus* line DH12075 (Dr Gerhard Rakow, Agriculture and AgriFood Canada, Saskatoon Research Centre) was used for all experiments. DH12075 is derived from a F_1 cross between the French cultivar Cresor and the Canadian cultivar Westar.

All experiments were carried out in growth chambers at the Phytotron Facility, College of Agriculture, University of Saskatchewan. Two seeds were sown in each of 25 to 30 four litre pots filled with Rediearth (W. R. Grace and Co. Canada Ltd., Ajax, Ont.). At the two-leaf stage, the seedlings were thinned to one per pot. Control growth conditions were; 16:8 hr, 23°:18°C, day:night cycles and 230-300 $\mu E\ s^{-1}\ m^{-2}$ at canopy level, depending upon height of the plants. Planting dates for the first and second repetitions of the growth chamber experiments were 29 September and 12 December, respectively.

When about 50% of the plants were flowering, the plants were randomly divided into one-week high temperature stress (1WHTS), two-week high temperature stress (2WHTS) and control groups. The HTS regime was: daytime temperatures ramped at 2°C per hr, from 23°C to 35°C over six hours, maintained at 35°C for four hours then

ramped back down, at 2°C per hour, to 23°C for the remaining six daylight hours. Night temperatures remained constant at 18°C for eight hours. After one or two weeks of HTS, the plants were returned to standard growing conditions until desiccation. To determine the effect of HTS on flower development the highest open flower on each inflorescence was tagged daily with a piece of dated tape. This allowed easy identification and tracking of those flowers exposed to HTS as well as the siliques and seeds that developed from the 3-6 flowers produced per inflorescence each day. At maturity, desiccated siliques were collected and enumerated for each plant for each day of flowering. Siliques containing seeds were collected and enumerated according to the day the flower opened, i.e., parthenocarpic siliques were not included in the silique data. Flower number and silique number data (totals from both terminal and lateral inflorescences) were each pooled for both repetitions of the experiment and adjusted (normalized) to 51 plants. The two repetitions of the experiment resulted in a total of 60 control, 55 1WHTS and 51 2WHTS plants. Seed number and weight were determined for 10 randomly selected plants from each repetition.

Three to six *B. napus* flowers reach anthesis sequentially along an inflorescence each day. Thus a one or two week HTS exposes flowers over a range of developmental stages to the stress treatment. By tagging the highest open flowers daily, the effects of the one- or two- week HTS treatments on flower, silique and seed production could be observed for the whole plant population.

Throughout the experiment, the position of each plant within the growth chamber was randomised weekly and the plants were kept well watered to prevent any effects associated with drought stress. Each plant was fertilized once prior to flowering with 50 mL of 2.5 g L^{-1} of 20/20/20 fertilizer, applied to the soil. Humidity, soil and leaf water potential were not measured. Humidity was only moderately controlled and was probably near 30%.

3.2.2 Pollen viability and nuclei staining

Pollen was stained using fluorescein diacetate (FDA) and the epifluorescence observed (Heslop-Harrison and Heslop-Harrison, 1970) using a Zeiss Axioplan microscope. Flowers of different ages were collected on specified days from control and

HTS-treated DH12075 plants and the pollen from all anthers used to determine viability. Flowers reaching anthesis on the day of collection, or one or two days prior to collection, were designated as 0-, 1- and 2-Day old flowers, respectively. Mature flower buds destined to reach anthesis the day following collection were also collected and designated -1 Day old flowers. Pollen was collected during the middle of the light cycle. On each collection day single flowers from three different plants for each age group were collected.

Mature pollen from three HTS-treated and three control plants was collected after one and four days of HTS and stained using 2,4-diamidino-2-phenylindole (DAPI: Pechan and Keller, 1988). Nuclei were visualised using a Zeiss Axioplan microscope with a DAPI epifluorescence filterset (Pechan and Keller, 1988). Data were pooled to give the number of nuclei observed for each treatment group.

3.2.3 In vitro *pollen tube growth*

Pollen from control or HTS-treated plants was collected and germinated using Hodgkins and Lyons medium containing 9% sucrose and 13% polyethylene glycol (MW 4000) (Rao et al., 1992). The pollen was incubated in light and high humidity for four hours at either 23°C or 35°C. Pollen tubes were counted and photographed using a Synsys digital camera (Photometrics) attached to a Zeiss Axioplan microscope. The digital images were processed using the MetaVue programme by Universal Imaging Group (Downingtown, PA, USA).

3.2.4 Reciprocal crosses between HTS and control pollen and pistils

Immature flowers (those due to open the next day, -1-Day) from both control and HTS-treated plants were emasculated by hand. Manual pollination was performed the day after emasculation, using pollen from anthers of either control or HTS-treated plants eight hours into the light cycle on the fourth day of exposure to HTS. Thus, developing flowers were exposed to four days of the HTS treatment prior to pollination. After pollination, plants remained at either control or HTS conditions until the end of the HTS treatment, when all plants were grown under control conditions. Pistils were collected approximately 24 hours after pollination and fixed in 1:3 glacial acetic acid:70% ethanol

prior to preparation for scanning electron microscopy (SEM) as described by Hill and Lord (1987). Pollen germination and pollen tube growth in the ovary were observed with a Phillips 505 SEM (Hill and Lord, 1987).

Seven or eight pistils from each of the reciprocal crosses were left to develop for ten days following pollination (final three days of HTS followed by one week of control temperatures) before being fixed and stored under ethanol.

3.2.5 Detection of HSP mRNAs

Reverse Transcriptase PCR (RT-PCR) was used to determine the presence of *HSP* mRNAs in pollen, unpollinated pistils and leaf tissue. Mature pollen was collected from control plants by agitating anthers, excised from freshly opened flowers, in 700 µl of 10% sucrose. Pollen was sedimented by centrifugation at 100 x g for two min and excess sucrose drawn off by pipette. The pollen pellet was resuspended in 100 µl of 10% sucrose and aliquots incubated at 23°C for 60 minutes or at 35°C for 30 or 60 minutes. Immediately after incubation the pollen was ground to a powder under liquid nitrogen and total RNA was extracted using a Plant RNeasy kit (QIAgen, Mississauga, ON). HTS pollen was collected from freshly opened flowers of HTS-treated plants and total RNA extracted immediately. Leaf disks and the unpollinated pistils of unopened flower buds from control and HTS-treated plants were also collected, ground to powder under liquid nitrogen and total RNA extracted using the Plant RNeasy kit. All tissue was collected from plants in the middle of the light cycle during the fourth day of HTS.

RT-PCR was performed on 128 ng of RNAse-free DNAse-treated total RNA using a One Step RT-PCR kit (QIAgen). Primers and dideoxynucleotide-tailed competimers (courtesy of Fatma Kaplan, University of Florida) were used at a 2:4 ratio to amplify a 309 bp fragment of 18S rRNA as an internal control for RT-PCR quantification (Sung et al., 2001). Primers specific to *B. napus HSP17.6*, *HSP70* and *HSP101* were designed using sequences obtained from a *B. napus* EST database (courtesy of Drs Andrew Sharpe and Derek Lydiate; Agriculture and AgriFood Canada, Saskatoon Research Centre; email brassica_est@em.agr.ca for further information on the EST library). Primers were designed to amplify conserved regions of the HSP genes, as determined by alignments with *A. thaliana* orthologues. The *Arabidopsis*

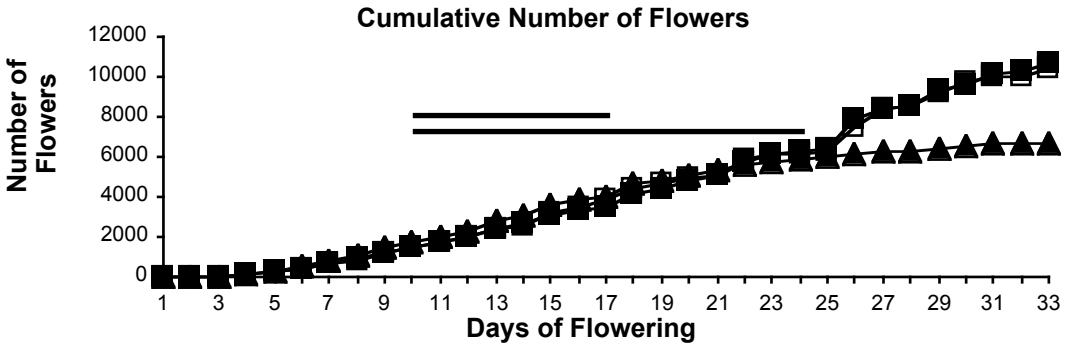
HSP17.6 and *HSP101* orthologues are heat inducible, while the *B. napus HSP70* shared greatest identity (~85% at the nucleotide level) with constitutively expressed *Arabidopsis* mitochondrial *HSC70-1* (gene location At4g37910). A mitochondrial *HSP70* was chosen because gametophytes have a high metabolic rate. For *HSP17.6* and *HSP101*, the 18S rRNA internal control RT-PCRs were performed using a separate aliquot of the same total RNA. The primers were gene specific and the amplified sequences did not contain intervening introns or intragenic spacers. Equal volumes of *HSP17.6* or *HSP101* and 18S rRNA reactions were loaded into one well of a 1.5% agarose gel and quantification performed by comparing the *HSP* RT-PCR product band intensities to that of the 18S rRNA RT-PCR product. RT-PCRs were performed with both *HSP70* and 18S rRNA primers in the same RT-PCR. Agarose gels were photographed and band intensity determined using a Quantity One GelDoc system (BioRad).

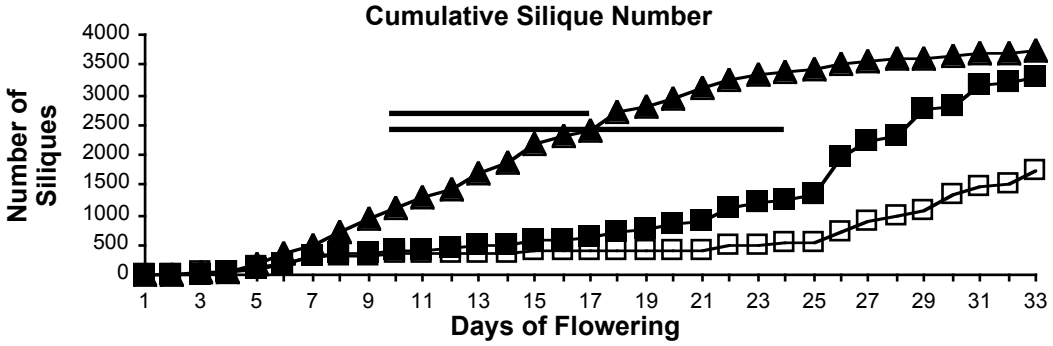
3.3 RESULTS

3.3.1 HTS adversely affected B. napus *gametophyte function and induced embryo abortion*

Quotidian tracking of flowers exposed to HTS allowed analysis of the effects of HTS on a wide range of flower and silique developmental stages. HTS treatment did not affect the numbers of *B. napus* flowers produced (Fig. 3.1A) but did have a severe detrimental effect on silique and seed production during the HTS period (Fig. 3.1B-D). Flower production in one-week high temperature stressed (1WHTS) and two-week high temperature stressed (2WHTS) plants continued throughout the experiment at the same rate as control plants (Fig. 3.1A). Flowering in the control plants declined to minimal levels after 22 days whereas in 1WHTS and 2WHTS plants flower production remained constant until the end of the experiment. The HTS-treated plants produced a significantly higher number of lateral inflorescences resulting in the constant production of flowers throughout the experiment (Table 3.1). The significantly greater number of inflorescences produced by the HTS-treated plants was responsible for the increased production of siliques and seeds in the last seven days of the flowering, but only after the cessation of the HTS treatment (Fig. 3.1).

Figure 3.1 The effects of HTS on flowering, silique production and seed set in *B. napus* DH12075 plants.
The HTS period started on the 10th day of flowering and proceeded for 7 (1WHTS) or 14 days (2WHTS) (indicated by short and long horizontal lines, respectively) before the plants were returned to standard growing temperatures. The total number of flowers and siliques, pooled from two repetitions of the experiment (25-30 plants for each repetition) are shown. Control plants (▲; n=60), 1WHTS (■; n=55), and 2WHTS (□; n=51) treatments are indicated, normalised to 51 plants for silique and flower number. Seed number and seed weight data are from 10 randomly selected plants from each repetition for each treatment. A) Cumulative number of flowers, B) cumulative number of siliques, C) cumulative seed number and D) cumulative seed weight over 33 days of flowering.


Cumulative Number of Flowers
Number of Flowers
12000
10000
8000
6000
4000
2000
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33
Days of Flowering


Cumulative Silique Number
Number of Siliques
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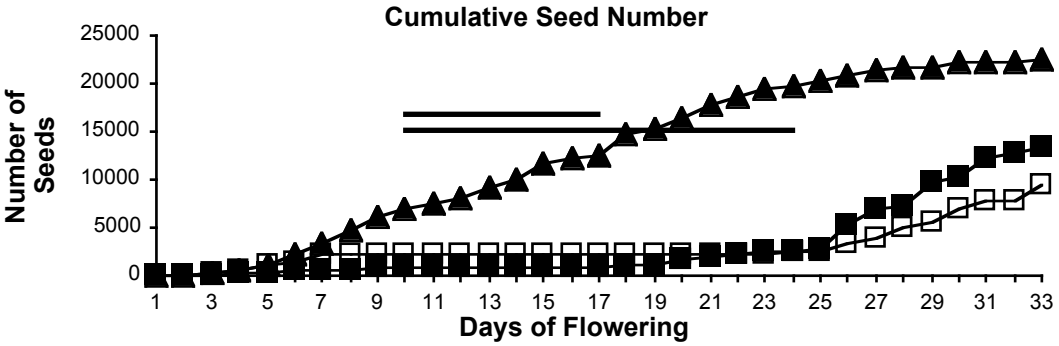
3500
3000
2500
2000
1500
1000
500
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33
Days of Flowering


Cumulative Seed Number
Number of Seeds
25000
20000
15000
10000
5000
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33
Days of Flowering

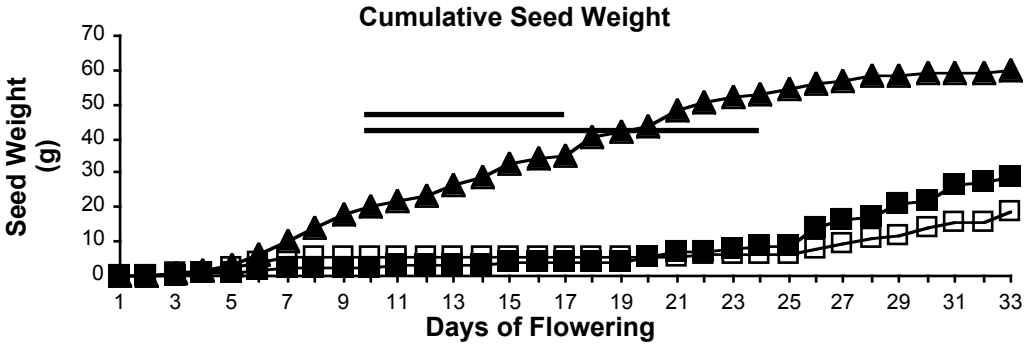

Cumulative Seed Weight
Seed Weight (g)
70
60
50
40
30
20
10
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33
Days of Flowering

Table 3.1 Mean number of lateral inflorescences produced by control, 1WHTS or 2WHTS treated *B. napus* DH12075 plants.
Using an ANOVA a significant difference in the number of lateral inflorescences produced by the three temperature treatments ($p < 0.001$). LSD showed significant differences between the number of laterals growing on control and HTS-treated plants. Data are based on one repetition of the experiment, but are representative of both repetitions.

	Control	1WHTS	2WHTS
mean number of inflorescences	3.3	5.9[a]	5.7[a]
n	30	25	26

a = significant at p □ 0.001

HTS had an adverse effect on post-pollination events affecting the development of seed and/or siliques. Flower development into siliques and seeds was inhibited by the HTS in those flowers fertilised up to 4 days prior to the initiation of the HTS (Fig. 3.1B-D). On the first day of the HTS (day 10 of flowering), the disruption in seed and/or silique development occurred in those flowers that had opened (and presumably were pollinated) on days 6-9 of flowering. That is, developing embryos or seeds up to four days post-pollination were sensitive to HTS such that an inhibition of seed production was observed in siliques that developed from flowers that opened on day 6 (Fig. 3.1C and D).

The decreased level of silique and seed production continued in those flowers that opened several days after the removal of the HTS suggesting that gametophyte development was adversely affected by HTS (Fig. 3.1). If the disruption of seed and/or silique development were the sole reason for decreased seed production during HTS then seed and silique production would have restarted on the day the HTS was removed. Inhibition of seed production continued for eight (1WHTS) and two days (2WHTS) after the removal of the HTS stress (Fig. 3.1C); therefore, gametophyte development and/or function were irreversibly affected by the HTS.

B. napus inflorescences were unable to acclimatize during the HTS period. Seed production was reduced throughout the HTS period regardless of the duration of the HTS (Fig. 3.1C, D). After removal of the HTS, the rate of seed production increased rapidly in HTS-treated plants due in part to the greater number of lateral inflorescences produced by the HTS-treated plants.

HTS limited pollen viability but did not affect microgametophyte meiosis or mitosis. Overall pollen viability (mean of viability scores from one day before opening to two days after anthesis) was lower for HTS pollen compared to control pollen during the HTS period (Fig. 3.2). Overall pollen viability during the HTS treatment ranged from 30-70% during the HTS period and up to five days after the removal of the HTS treatment (12^{th} day) but returned to control levels (81%) by eight days after removal of the HTS treatment (15^{th} day).

A second trend in pollen viability was that 1 Day and 2 Day flowers exposed to HTS contained a lower percentage of viable pollen (58%) when compared with control

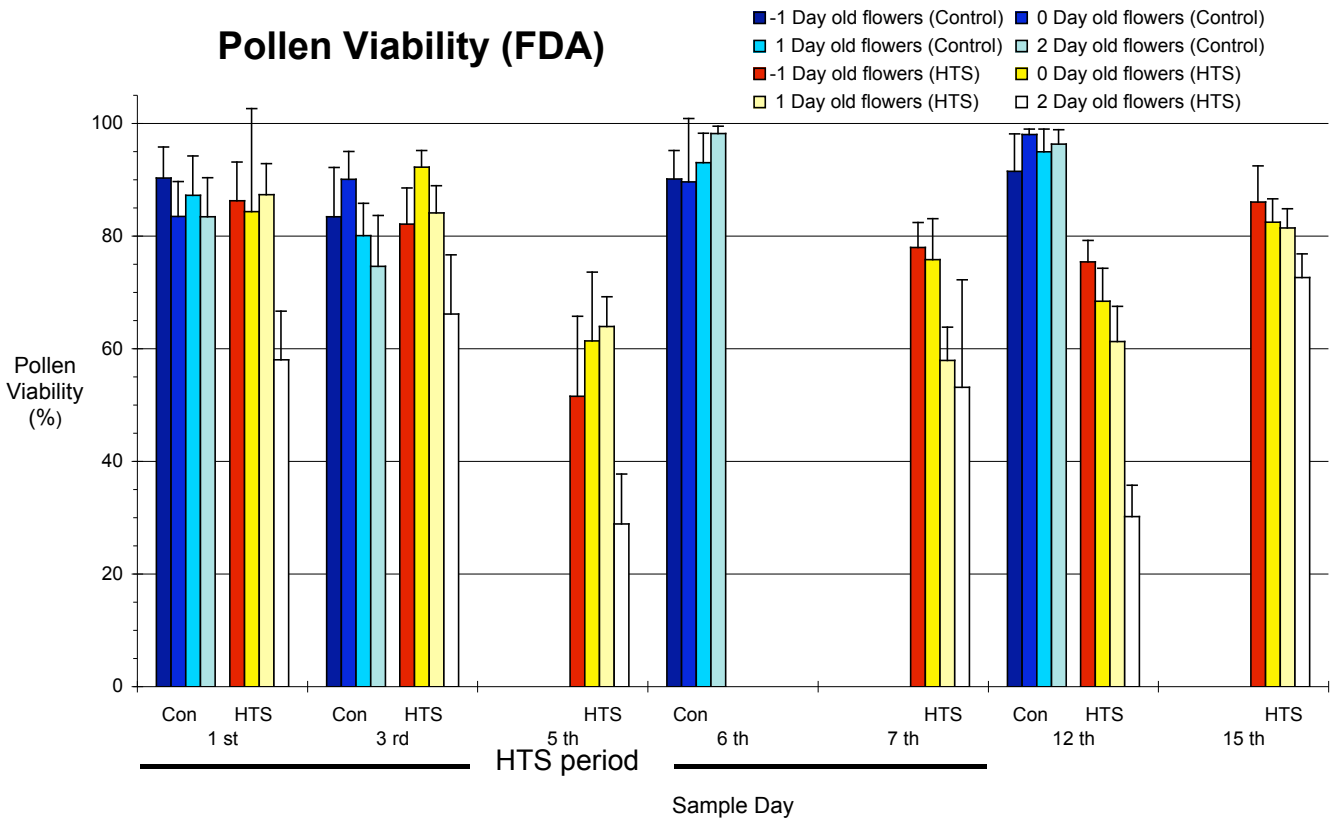


Figure 3.2 Viability of pollen from 1WHTS treated and control *B. napus* DH12075 plants.

Pollen viability was determined using FDA staining from -1, 0, 1 and 2 Day old flowers. HTS treatment was for 7 days with the 1st sample coincident with the 1st day of HTS (HTS). Flowers from heat stressed plants were collected at mid-day on the 1st, 3rd, 5th, and 7th day of HTS treatment and 5 and 8 days after the resumption of standard growing temperatures. Flowers from control plants were collected on the 1st, 3rd, 6th and 12th days of the experiment (Con). Bars represent the mean pollen viability while error bars represent the SE (n=3)

flowers of the same age (86%), or –1 Day or 0 Day flowers exposed to HTS (78%). The overall levels of pollen viability suggest that tissues surrounding the pollen provided some level of protection in maintaining pollen viability during HTS.

B. napus pollen grains are trinucleate so the presence of three nuclei in mature pollen (as observed by DAPI staining) suggests that microspore mitosis (and probably meiosis) had occurred in some microspores indicating that normal development of a subpopulation of pollen had occurred in the anther. The same ratio of the numbers of nuclei was observed in mature pollen grains from both HTS-treated and control plants (Table 3.2). Pollen that had matured prior to the HTS treatment had a similar ratio of 0:1:2:3 nuclei to that of control pollen after eight hours of HTS. Pollen collected from freshly opened flowers on the fourth day of the HTS treatment also had a similar ratio to control pollen indicating that microspore meiosis and mitosis were unaffected by HTS (Table 3.2).

3.3.2 HTS affects in vitro *pollen germination and* in vitro *pollen tube growth*

Pollen taken from plants exposed to four days of HTS had lower *in vitro* germination rates than pollen from control plants (17.5% vs. 59.2%, respectively). Pollen from HTS-treated plants had lower *in vitro* germination rates when germinated at either 23°C or 35°C.

High temperatures during *in vitro* germination also had a detrimental effect on pollen tube growth. Abnormal pollen tubes were produced by 0 Day pollen from both control and four-day HTS-treated plants when germinated *in vitro* at 35°C for three hours, but not at 23°C (Fig. 3.3A-D). In comparison, pollen tubes from control and HTS pollen that developed *in vitro* at 35°C for three hours were thinner and more convoluted than those that developed at 23°C (Fig. 3.3C,D). Pollen tubes from control pollen growing towards the micropyle of an ovule in a HTS plant over 24 hours of a HTS cycle had a normal morphology (Fig. 3.3E).

3.3.3 Both male and female gametophyte function are adversely affected by HTS

SEM observations revealed that HTS and control pollen germinated and extended pollen tubes along the septum and towards the micropyles in both HTS and

Table 3.2 DAPI staining of pollen from HTS-treated and control grown plants.
Plants were grown and HTS-treated as described in Material and Methods. Mature pollen grains were collected from at least one one-day-old, fully opened flower from each of three plants. Pollen collection was on the first or fourth day of the HTS, in the middle of each light cycle, after the ramping up of temperature and two hours at 35ºC (for HTS-treated plants) or 8 hours at 23ºC for control plants. The ratio of pollen grains containing 0, 1, 2 or 3 nuclei are shown, data are pooled from three individual plants.

	Control grown plants (n =3)	HTS-treated plants (n=3)
# of nuclei	0 : 1 : 2 : 3	0 : 1 : 2 : 3
8 hours of HTS	108 : 7 : 26 : 161	88 : 4 : 11 : 207
4 days of HTS	44 : 4 : 7 : 295	46 : 2 : 25 : 211

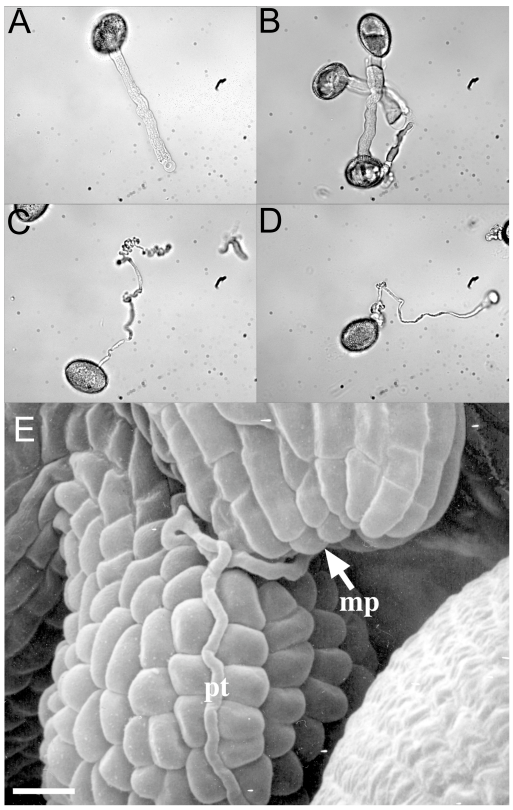


Figure 3.3 *In vitro* and *in vivo* pollen tube growth.
Pollen from both HTS and control grown plants were germinated *in vitro* at either 23°C or 35°C for three hours. Mean pollen grain length was 42.8 μm ± 2.00. A) Control pollen germinated at 23°C. B) HTS pollen germinated at 23°C. C) Control pollen germinated at 35°C. D) HTS pollen germinated at 35°C. E) SEM of Control pollen tube (pt) approaching the micropyle (mp) of a HTS ovule. Bar = 20 μm

control pistils (Table 3.3). The ratio of pollen tubes to ovules was lower for HTS pollen than for control pollen, however (Table 3.3). Some of the pollen grains from HTS plants that successfully germinated *in vivo* produced pollen tubes with normal morphology and function. This suggests that HTS during pollen development resulted in a reduction in the percentage of germinating pollen rather than affecting pollen tube growth and function after germination.

Reciprocal crosses between HTS and control pollen on HTS and control pistils show that HTS affected both pollen and pistil functionality. Seed set was reduced by 88% when pollen donor plants were HTS-treated and by 37% when the emasculated receptor plants were HTS-treated even though the number of pollen tubes per ovule in Control pollen x HTS pistils was similar to the number in Control pollen x Control pistil crosses, as observed by SEM (Table 3.3). The reduction in silique and seed production in Control pollen x HTS pistil crosses when compared to Control pollen x Control pistil crosses was due to reduced functionality of female gametophytes in the HTS-treated receiver plants. Likewise, the reduction in silique production in HTS pollen x Control pistil crosses was due to reduced pollen functionality.

Some reciprocal crosses were allowed to mature after the removal of the HTS to determine the effect of HTS on fertilisation and embryo development (Table 3.3, Fig. 3.4). Control pollen x Control pistils produced 19 elongated seed-containing siliques from 24 crosses (79%); HTS pollen x Control pistils, 53%; Control pollen x HTS pistils, 68%; and HTS pollen x HTS pistils, 8%. Seed production success was not related to initial silique elongation when one or both parents were HTS-treated. While Control pollen x Control pistil crosses produced no parthenocarpic siliques, 6%, 3% and 40% of the elongated siliques were parthenocarpic in HTS pollen x Control pistil, Control pollen x HTS pistil and HTS pollen x HTS pistil crosses, respectively. Abortion of the pistil without elongation was also quite common if one or both of the parents were HTS-treated (Fig. 3.4, arrows).

Seed production decreased as the number of parents treated with HTS increased. Control crosses produced 17.4 seeds per silique compared with 10.9, 3.9 and 0.5 seeds per silique produced by Control pollen x HTS pistil, HTS pollen x Control pistil and

Table 3.3 HTS and control pollen crosses with HTS or control pistils.
Pollen from control (Con pol) or HTS (HTS pol) grown flowers was used in reciprocal crosses with emasculated control (Con pis) or HTS (HTS pis) flowers. Using SEM, pollen germination, pollen tube growth in the ovary, and the number of ovules were determined in six dissected pistils for each cross. Further reciprocal crosses were allowed to develop for 10 days after pollination resulting in seven to eight siliques (replication 1) and 18-25 siliques (replication 2). The mean percentage of aborted pistils, parthenocarpic and seed producing siliques within a treatment, and the mean number of seeds per silique were determined. The pooled results from two replications of the experiment are shown.

Cross	Con pol x Con pis	HTS pol x Con pis	Con pol x HTS pis	HTS pol x HTS pis
Pollen germination observed?	Yes	Yes	Yes	No*
Ratio pollen tubes:ovules	35:129	9:123	48:105	3:158
% aborted pistils	21	41	29	52
% parthenocarpic siliques	0	6	3	40
% seed containing siliques	79	53	68	8
Seed production (mean # seeds/silique)	17.4	3.9	10.9	0.5
% seeds per silique relative to Con pol x Con pis	100	22.4	62.6	2.9

* pollen germination not observed, but inferred to have occurred rarely due to the presence of pollen tubes in the ovary.

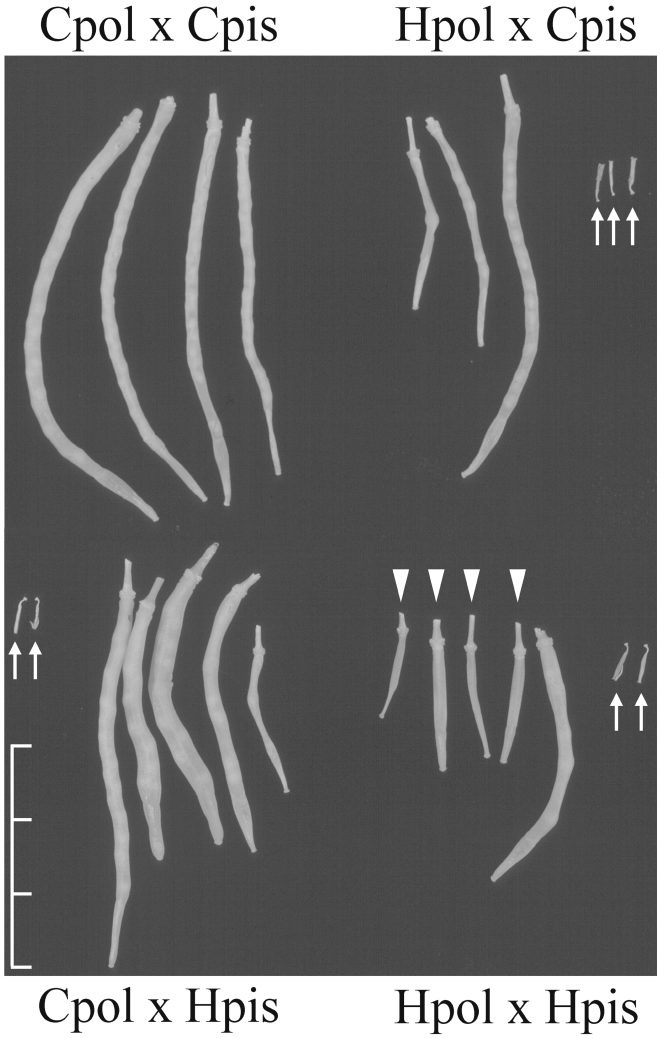


Figure 3.4 Aborted pistils, parthenocarpic siliques and filled siliques produced when neither, one or both parent plants were HTS-treated.
Emasculated HTS or control grown flowers were pollinated with pollen from plants grown under HTS or control temperatures after four days of HTS treatment. The siliques were allowed to grow for 10 days after pollination, the first three of which were under HTS conditions. The developing siliques were fixed and stored under ethanol. Pistils aborting prior to silique formation are indicated with arrows. When one or more of the parent plants were HTS-treated, mean seed number per silique was reduced. When both parents were HTS-treated, parthenocarpic siliques were produced (arrowheads). These siliques elongated but did not contain seeds. No parthenocarpic siliques were produced in Control pollen x Control pistil crosses. Seven or eight flowers were pollinated for each cross (some siliques were taken for dissection and are absent from the photograph). Scale bar indicates 10 mm graduations.

HTS pollen x HTS pistil crosses, respectively (Table 3.3). Both male and female gametophytes were adversely affected by HTS.

3.3.4 HSP transcription in pollen and pistils.

B. napus HSP17.6, *HSP70* and *HSP101* transcription in gametophytic tissues under HTS conditions was determined using RT-PCR amplification from total RNA. It has been reported that the presence of HSPs improved thermotolerance (Hong et al., 2001; Malik et al., 1999). Determining HSP gene expression patterns may help explain why differential thermotolerance was observed between male and female gametophytes, therefore.

HSP17.6 expression was observed only in pollen from HTS-treated plants (Fig. 3.5A). No expression was induced in mature control pollen, control pistils or control and HTS-treated leaves.

HSP70 expression was not observed in pollen but was present in control and HTS-treated pistils and leaves (Fig. 3.5B). The level of *HSP70* transcript in the HTS pistils was slightly higher than that observed in control pistils but HTS did not appear to increase transcription of this gene in leaves.

HSP101 expression was observed in all tissue types except mature pollen (Fig. 3.5C). HTS pistils had a higher amount of *HSP101* transcript compared with control pistils. In contrast there was no increase in *HSP101* mRNA in HTS leaves compared to control leaves.

3.4 DISCUSSION

HTS reduced *B. napus* DH12075 seed production not by reducing the number of flowers produced but by reducing micro- and mega-gametophyte fertility and disrupting post-pollination events in fully opened flowers. The outcome was an overall reduction in silique and seed production during the HTS. Although silique production in 1WHTS plants almost recovered to control levels by the end of the experiment, this would not occur in the field in Saskatchewan where the short growing season curtails the maturation of the late-developing siliques.

I was primarily interested in the overall effect of HTS on silique and seed production. Consequently, tracking the highest fully open flower every day in the initial

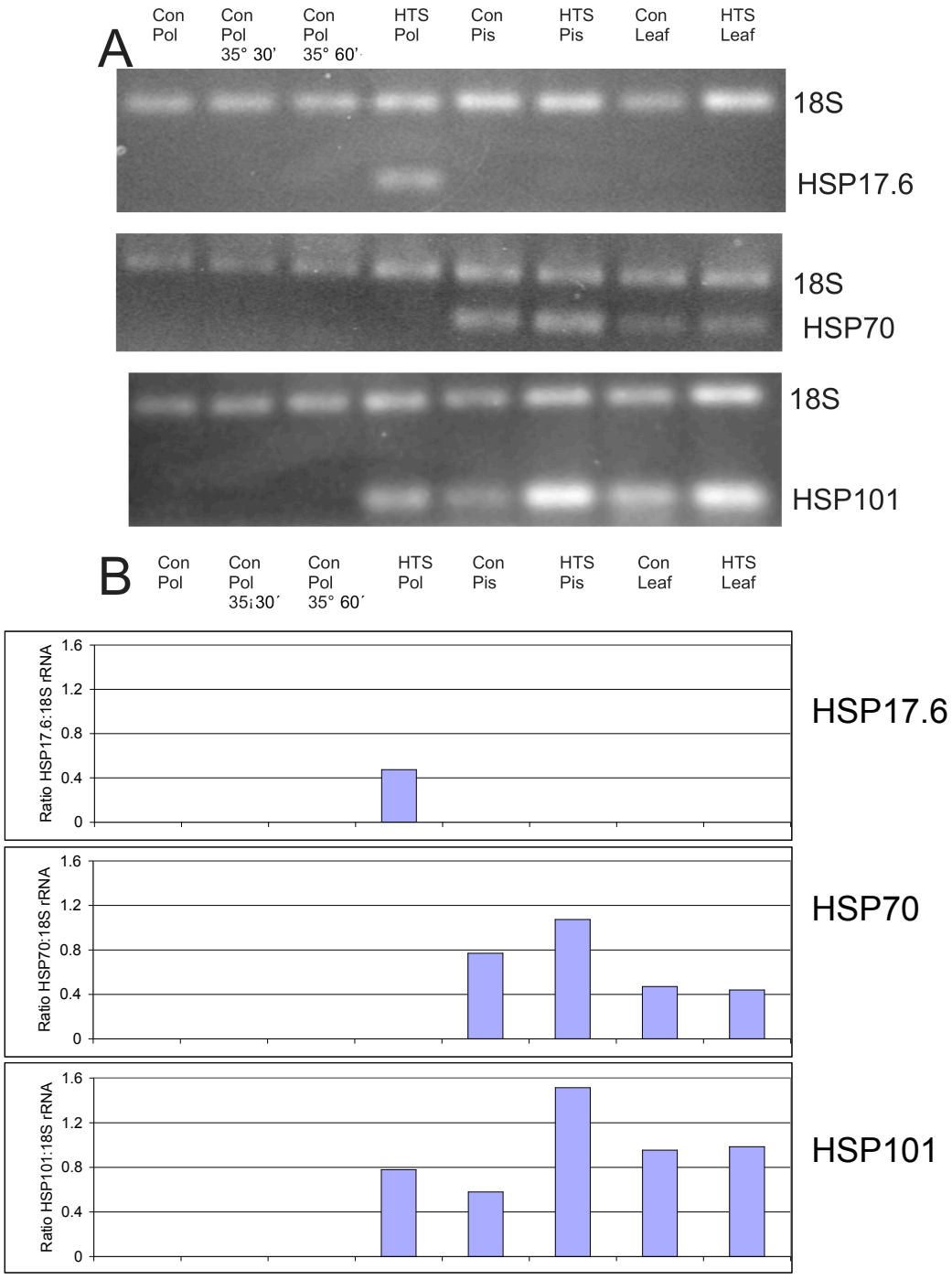


Figure 3.5 HSP mRNA in *B. napus* pollen, pistils and leaves.
A) RT-PCR amplification of *HSP17.6*, *HSP70* and *HSP101* from control and HTS-treated pollen, pistil and leaf total RNA. RT-PCR of 18S rRNA was carried out in parallel as an internal control. B) Specific gene: internal control ratios were calculated and are shown below each gel.

experiments was required to determine which tissues were affected by HTS. Determination of the exact developmental stage(s) adversely affected by HTS would have been difficult as all stages of development, from developing flowers to maturing siliques, were exposed to the HTS and each flower/silique passed through several stages of development during the 7 or 14 days of the stress. Some differentiation between stages at the beginning and ends of the HTS could be made, however.

Reduced microgametophyte function as a result of HTS has been observed in various species including *Capsicum annuum* (L) (Erickson and Markhart, 2002) *Linum usitatissimum* (Cross et al., 2003), *Lycopersicon esculentum* (Peet et al., 1998; Sato et al., 2002), *P. vulgaris* (Weaver et al., 1985), *T. aestivum* (Saini and Aspinall, 1982) and *Z. mays* (Herrero and Johnson, 1980). Likewise, adverse effects on megagametophyte development and/or function due to HTS have been observed in *B. napus* (Polowick and Sawhney, 1988), *L. esculentum* (Sato et al., 2002) and *T. aestivum* (Saini and Aspinall, 1982). My observations show that reduced gametophyte fertility and disruption of seed development together resulted in the almost total abolition of seed production during the HTS period.

Reduced pollen germinability was probably the major cause of reduced HTS pollen fertility. Pollen that had developed under HTS conditions had lower germination rates than control pollen, when both were germinated *in vitro* at 23°C, suggesting that the reduced germination rate of HTS pollen was due to changes that occurred during microspore or pollen development and maturation, rather than HTS at the time of pollen germination. The HTS-induced changes did not affect microspore meiosis, tetrad development, pollen tube growth or guidance and, as determined by FDA staining, had only a small effect on pollen viability.

I assumed that the total number of pollen grains produced by HTS and control anthers was similar but that pollen failing to reach maturity due to a malfunction in meiosis or mitosis would not have been included in counts of viability or nuclei. Although HTS may have reduced the number of mature pollen present, a portion of the pollen population was able to develop correctly, undergoing meiosis, mitosis and remaining viable despite the HTS treatment. The amount of viable pollen produced by HTS plants should have been sufficient to fully fertilise the pistil. My observations of

gross pollen morphology suggest that the large reduction in pollen fertility was not a result of failure of the pollen to develop, undergo meiosis and mitosis or remain viable.

The reductions in seed and silique number during HTS could have been due to the adverse effects of HTS on silique development, especially the conversion from pistil to silique. My observations suggest that silique development is either independent of seed development or dependent on seed number rather than seed development being dependent on silique development. The development of numerous parthenocarpic siliques during HTS demonstrated that silique development is able to proceed, up to a point, independently of seed production. The number of seeds contained within a silique corresponds to the size of the silique (Fig. 3.4). The results of my work suggest that the primary cause of HTS-induced reductions in seed set are because of problems with pollination and/or fertilization rather than adverse effects on the initiation of silique development.

Reduced seed set in HTS-treated plants was not a consequence of reduced pollen viability. Sufficient quantities of viable pollen were available at anthesis in HTS plants to fully pollinate receptive pistils (Fig. 3.2), but almost total sterility was observed. Furthermore, pollination in *B. napus* occurs soon after anthesis (Dr G. Rakow, pers. comm.) when pollen viability was still high in HTS plants. Pollen viability, as determined by FDA staining, was not a reliable indicator of pollen fertility under these conditions.

Altered pollen tube morphology could contribute to reduced pollen fertility. *In vitro,* under HTS conditions (39°C for 30 min), germinating *Lilium longiflorum* (Thunb) and *Nicotiana tabacum* (L) pollen had altered pollen tube morphology and halted elongation (39°C for 30 minutes: Herpen et al., 1989). *B. napus* pollen germinating *in vitro* at 35°C for three hours had thinner pollen tubes with a convoluted morphology (Fig. 3C, D). Resumption of pollen tube growth was not examined but production of functional pollen tubes seems unlikely considering the thickness and apparent fragility of the ones produced under HTS. My SEM observations of *in planta* pollen tube growth suggest that the surrounding maternal tissues contributed to normal pollen tube growth even during HTS. I suggest that, *in planta*, pollen tubes may be physically constrained or protected by the surrounding pistil tissues. Furthermore, any production of a

synergid-derived pollen tube guidance signal(s) (Higashiyama et al., 2001; Wilhelmi and Preuss, 1997), if present in *B. napus*, was unaffected in HTS pistils, inferring that the synergids remained intact and functional during the HTS. Likewise, as demonstrated by their growth towards the micropyles of control ovules, the ability of HTS pollen to decipher a guidance signal remained intact. These interactions between maternal tissues and pollen tubes were independent of ovule viability. Altered HTS pollen tube morphology *in vitro* was not a good indicator of the situation *in planta*.

Unlike the situation in *L. longiflorum* and *N. tabacum*, where *in vitro* pollen tube growth stopped during a heat shock (39°C, 30 min) but resumed once the heat shock was removed (Herpen et al., 1989), changes to *B. napus* pollen developed under HTS conditions were irreversible. *In vitro* germination rates were the same for pollen from HTS-treated plants at 23°C and 35°C. Furthermore, if HTS-induced developmental damage to *B. napus* pollen was reversible, HTS pollen landing on HTS stigmas during the lower night time temperature (18°C), could have resulted in seed production during the 1WHTS or 2WHTS periods but did not. Likewise, the HTS pollen x Control pistil crosses were maintained at control temperatures and therefore should have produced as many seeds as Control pollen x Control pistil crosses if the processes inhibiting pollen fertility were reversible. These data suggest that the HTS-induced damage to pollen was irreparable even when pollen was allowed to germinate at non-HTS temperatures.

Microspore and/or pollen development are sensitive to HTS while mature pollen is more tolerant. My results showed that a relatively mild HTS (35°C for four hours) to developing pollen severely reduced pollen germination rates, with consequent reduced seed set. After 24 hours at 32°C, 36% of *Z. mays* pollen grains from tassels HTS-treated *in vitro* germinated compared with only 5% germination in pollen from tassels exposed to 38°C for 24 hours (Herrero and Johnson, 1980). Furthermore, *Lycopersicon esculentum* pollen that had developed during HTS produced fewer fruits than pollen from control plants (Peet et al., 1998; Sato et al., 2002). In contrast, ~50% of *B. juncea* ((L) Czern) mature pollen grains germinated after four or 24 hours at 45°C or 60°C (Rao et al., 1992) and *N. sylvestris* (Speg) and *Petunia hybrida* (Vilm) pollen germination frequencies were not affected by temperatures of up to 60°C for 48 h (Rao et al., 1995). Seed set was not affected when mature *B. juncea*, *P. hybrida* and *N. sylvestris* pollen

were incubated at either control temperatures or 60°C for 24 or 48 hours (Rao et al., 1992; Rao et al., 1995). *B. napus* plants HTS-treated at the early pod stage were more thermotolerant than plants treated at the early flowering stage (Angadi et al., 2000; Morrison, 1993). Differences in thermosensitivity of mature pollen from monocotyledonous and dicotyledonous plants may exist but further studies are required before conclusions can be drawn.

Mature *B. napus* pollen was more thermotolerant than developing pollen as only a small reduction in viability was observed in HTS-treated mature pollen grains compared to microspores that had developed under five days of HTS (Fig. 3.2, 1st vs. 5th days of HTS). These results are similar to those in *C. annuum* (Erickson and Markhart, 2002), *P. hybrida*, *N. sylvestris* (Rao et al., 1995) and *B. juncea* pollen (Rao et al., 1992).

Previous reports have shown that very little HSP synthesis occurs in mature pollen (Mascarenhas and Crone, 1996; Young et al., 2001). The increase in thermotolerance in mature *B. napus* pollen was not a result of increased *HSP* mRNA synthesis as mature *B. napus* pollen heat shocked for 30 or 60 minutes at 35°C did not synthesise a subset of *HSP* transcripts (Fig. 3.5). Mature pollen is already in a desiccated form with endogenous proteins already in a protected environment. I cannot exclude the possibility of HSP protein synthesis from pre-existing *HSP* mRNAs in my experiments, however.

Both *HSP17.6* and *HSP101* transcripts were detected in *B. napus* pollen developing under HTS conditions, suggesting that developing microspores are responsive to HTS. *Z. mays* pollen developing under HTS conditions produced elevated levels of small HSPs (Dupuis and Dumas, 1990) and *HSP101* mRNA and HSP101 protein (Young et al., 2001).

Higher levels of *HSP70* transcripts were observed in *L. esculentum* and *Z. mays* microspores developing under both control and heat stress conditions, but this transcript was not detected in mature pollen (Duck and Folk, 1994; Gagliardi et al., 1995). Similarly in *B. napus*, *HSP70* transcripts were not detected in mature pollen that had developed under HTS conditions, suggesting that expression of the *HSP70* homologue used in this experiment may be down-regulated as the pollen matures. Unlike reports in *Arabidopsis,* a slight increase in transcription of the mitochondrial *HSP70* has been

observed in HTS-treated *B. napus* pistils (Sung et al., 2001). Differences in stress conditions or tested tissues, between my work and that carried out in *Arabidopsis,* may explain why transcription of this orthologous *HSP70* gene was not reported in HTS-treated *Arabidopsis* (Sung et al., 2001). A very large increase in *HSP70* mRNA levels in HTS-treated tissues may not have been observed because the *HSP70* gene used in this experiment was a mitochondrial gene and may have been a HSC, i.e., constitutively expressed but not HTS inducible. In *B. napus*, both HSP70 and HSP101 are constitutively expressed in pistils and leaves with a higher level of expression in HTS-treated pistils. It is not clear as to why an increase in *HSP* mRNA was not observed during HTS treatment of *B. napus* leaf tissues. It is possible that leaf temperature because of increase to levels where *HSP* induction occurs due to transpirational cooling. Also, HSP mRNA levels do not necessarily reflect HSP protein levels during HTS (Dhaubhadel et al., 2002).

B. napus HSP17.6 was only expressed in pollen developing under HTS conditions, suggesting a thermoprotective rather than developmental function for this small HSP. In contrast, *Nthsp18P*, was expressed in *N. tabacum* pollen developing under non-stress conditions, suggesting a developmental role for this gene. Comparisons between sHSP expression patterns are not necessarily valid due to the diversity of the gene family, however (Waters et al., 1996).

My results from the reciprocal Control/HTS pollen x Control/HTS pistil crosses suggest that megagametophyte fertility was also affected by HTS. Susceptibility of *B. napus* megagametophytes to HTS has been reported previously (Polowick and Sawhney, 1988). In HTS-treated *B. napus* and *T. aestivum*, abnormalities in ovule morphology were observed using light microscopy (Saini et al., 1983), while in *L. esculentum*, reduced female fertility was inferred from reduced seed set (Peet et al., 1998; Sato et al., 2002). *Z. mays* differs from *B. napus, L. esculentum* and *T. aestivum* in that desiccation and loss of pollen viability are thought to be the primary cause of heat-induced yield reductions, while megagametophytes are unaffected (Dupuis and Dumas, 1990; Herrero and Johnson, 1980).

My data suggest that in *B. napus,* HTS may affect micro- and megagametophyte fertility in a synergistic manner, since the reduction in seed set in the HTS pollen crosses

was lower than the combined reduction in seed set when just one parent was HTS-treated (Table 3.3). That is, the observed seed set, as a percentage of the control crosses, was ~3% whereas we would expect to see ~14% if the effect were strictly additive (22.4% of 62.6%). Similar results have been reported in tomato (Peet et al., 1998) and wheat (Saini et al., 1983). In these latter two species and *B. napus*, it appears that HTS-induced sterility is due to a combination of both microgametophyte and megagametophyte dysfunction. A possible reason for this synergistic effect is a breakdown in the pollen-stigma adhesion mechanism occurring in HTS-treated plants. That is, if only one or the other parent is HTS-treated then pollen-stigma interactions are still able to proceed as normal. If both parents are HTS-treated then a reduction in the number of pollen grains able to adhere to the stigma may occur, further reducing the rate of pollen germination. Future careful experimentation, controlling the number of pollen grains deposited on the stigma, will be required before a synergistic effect can be confirmed. Another possible reason is the combined effect of carbohydrate limitation in HTS plants resulting in lowered photosynthate supply to the growing pollen tube from the stigma or a shortage of starch stored in the pollen grain. Limited carbohydrate might reduce fertilization rates if it occurred in combination but not be sufficient to inhibit function if occurring alone.

It is difficult to distinguish between the effects of megagametophyte sterility and embryo/seed abortion on seed production. My data suggest that HTS disrupts seed development post pollination. Disruption of embryo/seed development was not suggested as a cause of reduced seed set in reports of HTS treatment of tomato (Sato et al., 2002) or bell pepper (Erickson and Markhart, 2002) although it is possible to interpret some of the reductions in seed production in this way. In *B. napus*, the disruption of embryo/seed development was apparent in the lack of seeds produced from flowers that had been pollinated up to four days prior to the first day of the HTS (Fig. 3.1C). The total extent to which seed abortion affected seed production during HTS was not determined in this study. Disruption of embryo/seed development was not the sole cause of seed loss during HTS as inhibition of seed production continued two days after the HTS period in 1WHTS plants. If HTS-induced seed abortion was the sole contributor to failed seed set, then seed should have been produced immediately after (if

not before) the removal of the HTS. This was not the case, so I conclude that a combination of embryo/seed abortion and gametophyte sterility accounted for the reduction in seed set in *B. napus* under HTS conditions.

The production of parthenocarpic siliques when one or both parents were HTS-treated suggests that pollination initiates fruit formation. When one or both parents were HTS-treated pollination and/or pollen tube growth still occurred but the probability of successful seed formation was reduced. The increase in parthenocarpic silique formation when one or both parents were HTS-treated was due to the initiation of fruit development by pollination (or the events immediately following) followed by a combined failure of seed formation due to incomplete fertilisation or embryo abortion. The resulting partially elongated silique was parthenocarpic. Some work has shown that embryo development, rather than seed development is important for the early stages of fruit development (Vivian-Smith et al., 1999). Embryo abortion might prevent seed formation but still allow fruit development. The development of parthenocarpic fruit as a result of HTS-treatment has been reported in *C. annuum* (Erickson and Markhart, 2002), *B. juncea* (Rao et al., 1992) and *L. esculentum* (Peet et al., 1998). These data suggest that the HTS-induced production of parthenocarpic fruit is common to many species.

HTS caused almost total sterility in *B. napus* plants by reducing both microgametophyte and megagametophyte fertility. HTS effects on these tissues individually did not result in complete sterility, however. Other factors such as reduced pollen-stigma interactions or the observed inhibition in embryo/seed development could have contributed to reduced seed set in HTS plants. My findings suggest that investigations into the effects of HTS on reproduction must look further than the effects on pollen alone especially since HTS has now been shown to affect both gametophytes in three species; *B. napus*, *L. esculentum* and *T. aestivum*.

CHAPTER 4 TRANSGENIC BRASSICA NAPUS AND ARABIDOPSIS THALIANA PLANTS CONTAINING DNA CONSTRUCTS DESIGNED TO IMPROVE FLOWER THERMOTOLERANCE.

4.1 INTRODUCTION

High Temperature Stress (HTS) during flowering adversely affects seed set in several species including *Arabidopsis thaliana* L. (Kim et al., 2001), *Brassica napus* (Morrison, 1993; Polowick and Sawhney, 1987) and *Zea mays* (Herrero and Johnson, 1980; Schoper et al., 1987). As presented earlier, HTS during flowering had a greater effect on final yield of five crop species than did drought stress (see Chapter 2).

A range of species (both monocotyledonous and dicotyledonous) are affected at a similar time in development and in a similar manner, suggesting that HTS-induced reductions in seed set may be due to a common mechanism. In *Z. mays*, pollen dysfunction resulting from HTS is suggested as the primary cause of reduced seed set (Herrero and Johnson, 1980; Schoper et al., 1987). Dicotyledonous species appear to have a high level of pollen thermotolerance, however. For example, *Brassica juncea* L., *Nicotiana tabacum* L. and *Petunia hybrida* L. pollen were viable after incubation *in vitro* at 60°C for 12 hr (Rao et al., 1992; Rao et al., 1995), while in *B. napus* L., HTS caused a reduction in both male and female gametophyte fertility, reduced pollen germination rates (Chapter 3), and caused abnormal ovule development (Polowick and Sawhney, 1987; Polowick and Sawhney, 1988). HTS also adversely affected the ovules of *L. esculentum* (Peet et al., 1998) and *T. aestivum* (Saini et al., 1983).

Heat Shock Proteins (HSPs) are produced in cells exposed to a HTS (Nagao et al., 1986; Nguyen et al., 1989; Vierling, 1990). The role of HSPs is to protect cells from

heat-induced damage to proteins caused by high temperatures. Mutants lacking HSPs are unable to tolerate a mild HTS and subsequently die. For example, *Arabidopsis* seedlings lacking *HSP101* did not recover from a 120 minute HTS treatment at 45ºC, whereas wild-type seedlings did (Hong et al., 2000). *Arabidopsis tu8* mutants, which have an altered glucosinolate metabolism and do not produce HSP90 during HTS, were all killed by exposure to 32ºC for 21 days whereas wild-type plants survived this treatment (Ludwig Muller et al., 2000). Small HSPs are also necessary for acquired thermotolerance (Malik et al., 1999; Park and Bong, 2002). Overexpression of HSPs in transgenic plants may result in increased thermotolerance, therefore.

The function of orthologous HSPs is conserved between kingdoms. *Saccharomyces cereviseae hsp104* mutants became more thermotolerant when complemented by the orthologous *Arabidopsis*, soybean, wheat or maize *HSP101* (Lee et al., 1994; Schirmer et al., 1994; Young et al., 2001).

HSP gene transcription is regulated by Heat Shock Transcription Factors (HSFs). During HTS, HSFs form trimers which bind to the heat shock element (HSE) motif in the promoter of *HSP* genes, inducing transcription (Schoffl et al., 1998). Constitutive expression of *AtHSF3* in transgenic *A. thaliana* plants led to the constitutive expression of HSPs at control temperatures and a 2°C improvement in basal thermotolerance (Schoffl et al., 1998). The sublethal preconditioning heat shock that leads to acquired thermotolerance was not required in these transgenic plants.

I hypothesised that constitutive expression of *AtHSP101* in both *B. napus* and *Arabidopsis* would improve seed set in HTS-treated plants by improving flower (especially gametophyte) thermotolerance. Similarly, expression of *Arabidopsis HSF3* could also improve thermotolerance by inducing an increase in the transcription of a suite of thermoprotective *HSP* genes.

4.2 Materials and Methods

4.2.1 Design of constructs and production of transgenic plants

Initially I assumed that HTS caused flower abortion in *B. napus*. Accordingly, two DNA constructs were assembled and inserted into *B. napus* DH12075 plants. The *A. thaliana LEAFY* (*AtLFY*) promoter and ORF were obtained from Dr. D. Weigel (Salk

Institute) and the *A. thaliana HSP101* (*AtHSP101*) ORF from Dr. E. Vierling, (University of Arizona). Approximately 2 kb of a *HSP101* promoter (*HSP101B*) were amplified by PCR from an *Arabidopsis* BAC (F3D13; ABRC). Two constructs were assembled (Table 4.1, Fig. 4.1) using these components: *AtLFY*-promoter:*AtHSP101*-ORF (abbreviated as LPHO throughout this chapter) and *AtHSP101B*-promoter:*AtLFY*-ORF (HPLO). An *AtHSP101B*-promoter:*GUS* construct was also produced and is described in detail in Chapter 5 as *HSP101B*:*GUS*. The constructs described above were ligated into the *Agrobacterium* binary vector pCGN1558 (Calgene, California) and then inserted into *B. napus* DH12075 plants by hypocotyl transformation (Moloney et al., 1989) using *Agrobacterium tumefaciens* strain PC2760. Three HPLO and four LPHO plants, confirmed as transgenic by PCR amplification of the transgene, were crossed to produce hybrid seeds. Hybrid plants were HTS-treated in the same manner as the parental plant lines (see "Growth and treatment of transgenic plants" below). Sequencing of the gene fragments cloned above (and for *AtHSF3*, below) by the DNA Sequencing Facility, NRC/PBI, confirmed that the clones were error-free.

AtHSF3 was amplified from genomic DNA by Dr. Ashley Byun (this lab) using degenerate PCR primers based on the AtHSF3 protein sequence. The constitutive *EntCup4* promoter (supplied by Dr. B. Miki, AAFC, Ottawa) was used to control expression of the *AtHSF3* ORF (EntCupHSF) or the *AtHSP101* ORF (EntCupHSP). The EntCupHSP and EntCupHSF constructs and an *EntCup4*-promoter:*GUS*-ORF construct were inserted into the pGreen29 binary vector (Hellens et al., 2000). These three constructs, as well as the CaMV *35S*-promoter:*AtHSP101*-ORF construct (Dr. Lindquist, University of Chicago; 35S:HSP), were used to transform both *B. napus* (Moloney *et al*, 1989) and *Arabidopsis* (Pylatuik et al., 2003) via *Agrobacterium*-mediated transformation.

All the constructs in this work had the nopaline synthetase (nos) transcription termination sequence at the 3' end of the ORF. In addition to the constructs mentioned above, other transgenic plant material was supplied by various labs. Transgenic *B. napus* seeds containing 35S:GUS constructs from the binary vectors pRD420 and

Table 4.1 Primers for the manufacture of DNA constructs used in this study.
Promoters and ORFs were PCR amplified from plasmids containing the specified sequence or from *Arabidopsis* BAC DNA (ABRC). The component fragments below and intermediate plasmids in the construction of the transgenes were ligated into pBluescriptKS+ before transfer to an *Agrobacterium* binary vector. The CaMV *35S* and *EntCup4* promoters were restriction enzyme digested from plasmids and the resulting fragments used in ligations.

Component	5' primer	3'primer	Template	Size (kb)
***LFY* promoter**	**5'gcgggatccatttttcgcaaaggaaagtcg3'**	**5'gcgcgcctgcagaatctatttttctctctctc3'**	**PDW132**	**2.3**
***LFY* ORF**	**5'ggcgaattcatggaccctgaaggtttcacg3'**	**5'cgcctcgagctagaaacgcaagtcgtcgc3'**	**pIL8**	**1.3**
***HSP101* promoter**	**5'gcgcgatcctgtagagttgatacgaagttg3'**	**5'ggcctgcagcttcgattagccttttaaaatcc3'**	***At* BAC F3D13**	**2.0**
***HSP101* ORF**	**5'cgcgcgctgcagatgaatccagagaaattcacac3'**	**5'gcgcgtcgacttaatcctcgatcatttcctc3'**	**pAZ105**	**3.0**
HSF3	**5'cgcggatccatggaatcggttcccgaa3'**	**5'cgggaattcttatttcctctgtgcttc3'**	**Genomic DNA**	**1.4**
CaMV *35S* promoter	**-**	**-**		**0.3**
***EntCup4* promoter**	**-**	**-**		**0.5**

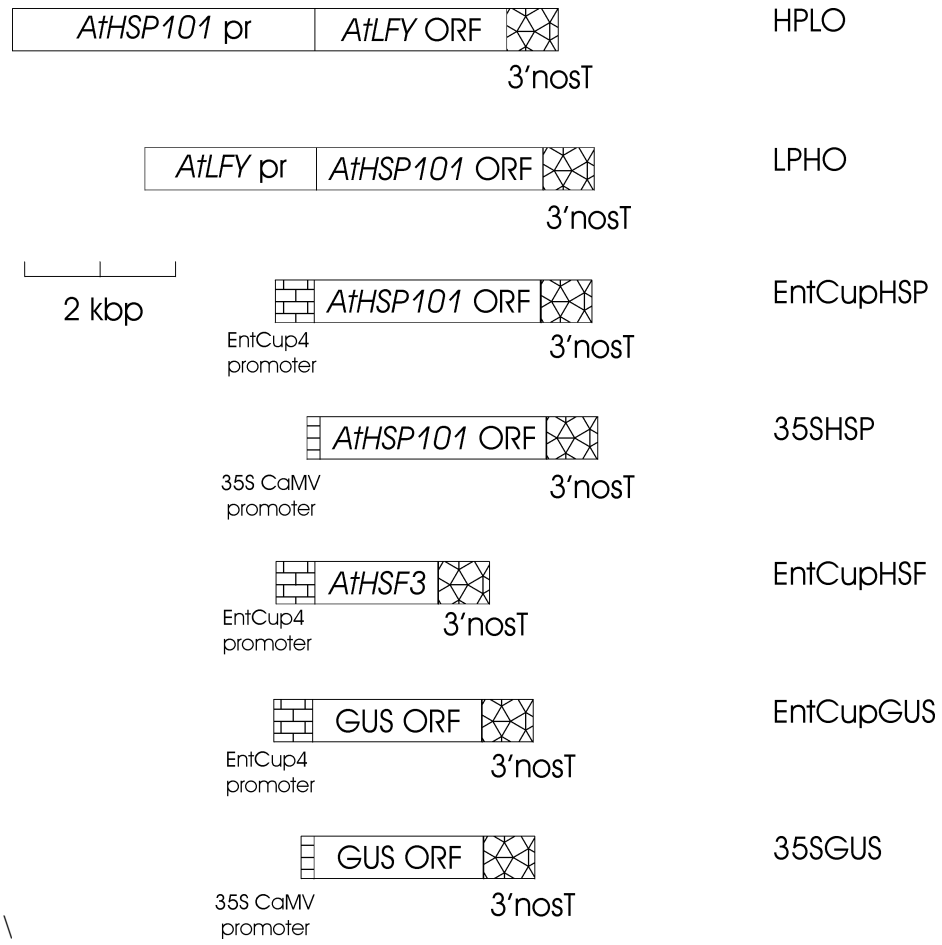


Figure 4.1 DNA constructs used in this project.

Diagrams showing constructs used in this project. HPLO and LPHO were inserted into pCGN1558 and EntCupHSP, EntCupHSF and EntCupGUS were inserted into pGreen29. The 35SHSP construct was obtained from Dr. Vierling and was in pBI121. All the constructs were inserted into *B. napus* DH12075, with the exception of 35SGUS, which was available in the lab in mature transformed seed from J. Hammerlindl (NRC/PBI). EntCupHSF and 35SHSP were also successfully inserted into *Arabidopsis*

pBI121 were provided by Joe Hammerlindl (National Research Council/ Plant Biotechnology Institute, Saskatoon). *B. napus* seed containing the *EntCUP3:GUS* construct were obtained from Dr. Miki (AAFC, Ottawa).

4.2.2 Determining transgene presence and activity.

DNA was extracted from T_1 plants (Sharp et al., 1988) and 100 ng used as the template for PCR amplification of the promoter, ORF or NPTII selectable marker. For the LPHO x HPLO hybrids, primers designed to specifically amplify both of the transgenes were used in the same reactions to simplify the identification of plants containing both constructs.

Total protein from unopened flower buds of untransformed (DH12075) and LPHO and leaf disks cut from HTS-treated DH12075 and HPLO plants were separated on a polyacrylamide gel and transferred to nitrocellulose membrane (BioRad) using a Protean II apparatus (BioRad). Polyclonal anti-AtLFY primary antibody (against the whole protein, supplied by Dr. D. Weigel, Parcy et al., 1998) and polyclonal anti-AtHSP101 primary antibody (against the N-terminal fragment of the protein, supplied by Dr. E. Vierling, Hong and Vierling, 2001), both at 1:1000 dilution, were used to determine the presence of transgene proteins. Secondary anti-rabbit IgG antibody conjugated with horse radish peroxidase and the Renaissance chemiluminescence kit (NEN) were used to visualise the primary antibody on the membrane.

RT-PCR amplification of *AtHSP101* in transgenic *B. napus* was performed using *AtHSP101* gene-specific primers and 100 ng of DNAse treated leaf total RNA. The One-Step RT-PCR kit (QIAgen, Mississauga, ON) was used to amplify a 180 bp fragment of the gene and a BioRad GelDoc system used to visualise the RT-PCR products after separation through a 1.5% agarose gel by electrophoresis.

4.2.3 Growth and HTS treatment of transgenic plants

For each transgenic *B. napus* line, two seeds were planted in each of ten 4 L pots filled with Rediearth (W. R. Grace and Co. Canada Ltd., Ajax, Ont.) and placed in growth chambers in the Phytotron Facility (College of Agriculture, University of Saskatchewan). Pots were thinned to one seedling per pot prior to the two leaf stage.

Standard growing conditions for all the experiments were 23°C/18°C 16 hr/8 hr day night cycles, 350-500 μE.m^{-2}.s^{-1} at the canopy level until flowering, which started 39-43 days after planting. Plants were well watered throughout and fertilised once with 50 ml of 2.5 g.L^{-1} 20/20/20 fertilizer just prior to flowering. Untransformed DH12075 plants were planted 10 days after the LPHO and HPLO lines so that flowering between lines was synchronous (see Results).

When approximately half of the plants had developed fully opened flowers they were randomly divided into two equal populations. One population from each line was transferred to a HTS treatment chamber to simulate a hot summer's day in Saskatchewan: daytime temperatures ramped at 2°C per hr, from 23°C to 35°C over six hours, remained at 35°C for four hours then ramped back, at 2°C per hour, to 23°C for the remaining six daylight hours. Night temperatures were the same as the control chamber at 18°C for eight hours. During the HTS treatment, leaf tissue samples were taken from the HPLO plants for total protein extraction to determine transgene activity (see below). After one week of HTS, the plants were returned to standard growing conditions until desiccation. Non-HTS-treated control plants were left at the standard growing conditions throughout.

To track flowers exposed to HTS conditions, a piece of tape was wrapped around the pedicel of the highest opened flower on the first and last days of HTS treatment. Cryovac bags were placed over the plants to prevent cross-pollination. Upon desiccation, siliques were collected, enumerated and grouped into pre-, during and post-HTS collections for each plant. Hybrid progeny from the LPHO x HPLO crosses were also tested for improved thermotolerance in the same way.

A modification of the HTS treatment was used to determine changes in thermotolerance of the 35SHSP and EntCupHSP *B. napus* lines. Individual transgenic plants were moved from standard growing conditions to the HTS chamber three days after the first flower opened. Each plant was exposed to the HTS for seven days and returned to the control growth chamber. Four plants from each line were HTS-treated, while four were kept at control temperatures throughout the experiment.

Ten lines each of 35SHSP and EntCupHSF *Arabidopsis* plants were HTS-treated in a manner similar to *B. napus*. Transgenic T_1 seeds were suspended in 0.1% agarose

and dispensed onto the surface of the Rediearth using a pipettor. Pots were thinned to one seedling per pot (6 pots per line) at the two-leaf stage and grown under standard conditions alongside the *B. napus* plants. An Aracon (Lehle Seeds, Texas) was placed around each plant the day the first flower opened and leaf tissue collected, frozen and stored at -80°C for RNA extraction. Next day the plant was either transferred to the HTS chamber for seven days or left in the control chamber (three plants per treatment per line). After HTS treatment, plants were returned to standard growing conditions until desiccation. Seed produced from plant was collected and weighed.

4.2.4 GUS staining of transgenic B. napus *tissue.*

Tissue from transgenic EntCupGUS and 35SGUS *B. napus* plants was fixed using acetone and vacuum infused with X-Gluc (see Sieburth and Meyerowitz, 1997).

4.3 RESULTS

4.3.1 Development of transgenic plants

The presence of the *NPTII* gene in transgenic plants was confirmed by PCR in 17 and 23 *B. napus* lines transformed with the LPHO and HPLO constructs, respectively (Table 4.2, Fig. 4.2). The presence of each of the transgenes was also confirmed by PCR amplification of the respective *Arabidopsis* ORF. For example, LPHO lines 13a, 16-23 and 25 were positive for *NPTII*, the *AtLFY* promoter and the *AtHSP101* ORF (Fig. 4.2).

Selected lines were tested for the presence of *Arabidopsis* HSP101 and LFY proteins using Western blots. AtHSP101 was detected in LPHO inflorescences but not DH12075 inflorescences or in HTS-treated *B. napus* leaves, showing that the antibody was specific to the *Arabidopsis* HSP101 and that the transgene was expressed in the inflorescences of transgenic lines (Fig. 4.3). Preliminary results indicated that AtLFY was present in the leaves of randomly selected HPLO lines but non-specific cross reactions between the antibody and unidentified proteins in the total protein extract prevented confirmation of AtLFY expression in transgenic lines.

A noticeable delay in the onset of flowering was observed in T_1 plants transformed with either construct (Fig. 4.4). In DH12075 plants (n=19) the first flower

Table 4.2 Summary of *B. napus* and *Arabidopsis* transgenic lines produced in this study.

The number of transgenic lines for each construct reported in this project are shown. The LPHO x HPLO hybrids were not produced as a result of transformation, so no vector is assigned to these lines. *B. napus* seeds from transgenic EntCup3GUS and 35SGUS plants were obtained from Dr. B. Miki (AAFC, Ottawa) and J. Hammerlindl (NRC/PBI), respectively. Dashes (-) indicate transformations not performed while a zero denotes unsuccessful transformations, with zero transgenic lines produced. n/a = not applicable

Transgene	Number of *B. napus* lines	Number of *Arabidopsis* lines	Binary vector	Construct manufacturer
LPHO	17	-	pCGN1558	L. Young
HPLO	23	-	pCGN1558	L. Young
LPHOxHPLO hybrids	100	-	n/a	n/a
EntCup4HSP101	2	0	pGreen29	L. Young
EntCup4HSF	0	10	pGreen29	L. Young
35SHSP	10	10	PBI121	S. Lindquist
35SHSF	0	0	pGreen29	L. Young
EntCup3GUS	T_1 seed (B. Miki)	n/a	n/a	n/a
EntCup4GUS	0	0	pGreen29	B. Miki/L. Young
35SGUS	T_1 seed with pRD420 and pBI121 (J. Hammerlindl)	n/a	n/a	n/a

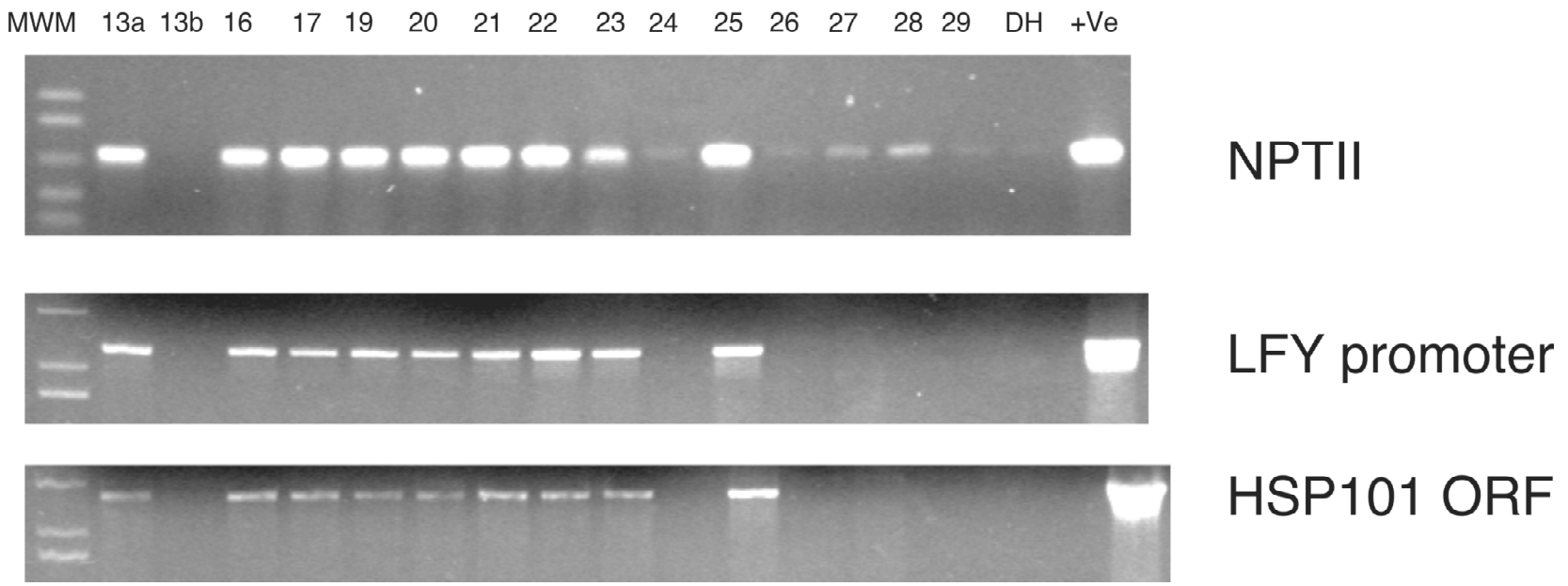


Figure 4.2 Confirmation of transformation in *B. napus* LPHO T_0 lines 13a - 29
PCR amplification of the *NPTII* ORF, *LFY* promoter and *HSP101* ORF showed the presence of the transgene in *B. napus* LPHO lines. MWM, 1 kb plus molecular weight marker; numbers identify independent plants rescued from tissue culture; DH is the DH12075 negative control; +ve is the PCR product amplified from 25 ng of the Ti plasmid used to transform the plants. Lines 13 and 13a were both subtended by the same mass of callus and were treated as independent plants.

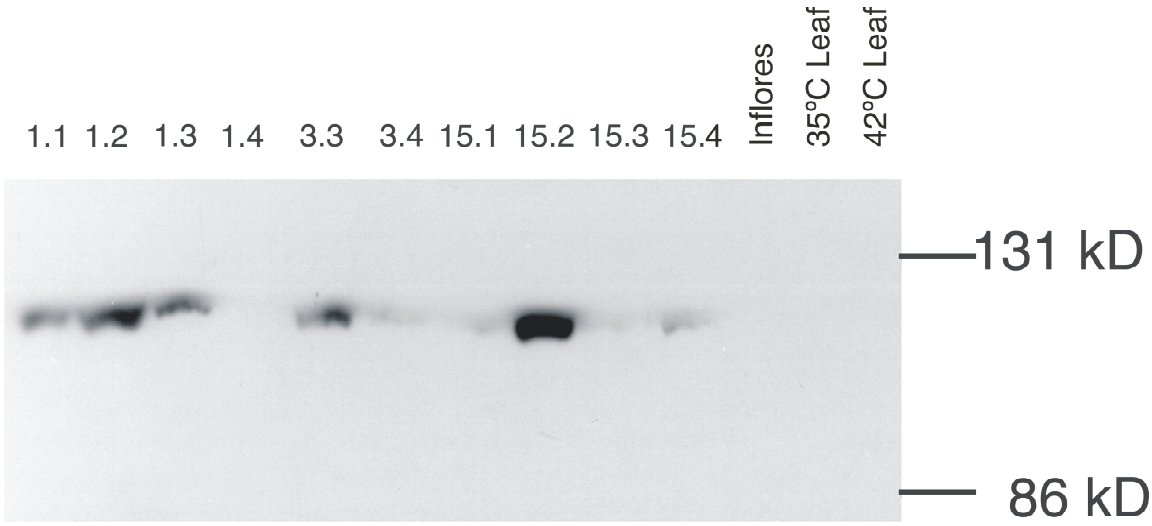


Figure 4.3 Western Blot of *B. napus* LPHO T_1 lines using anti-HSP101 antibody. *Arabidopsis* HSP101 was detected in total protein extracts from the inflorescences of T_1 transgenic *B. napus* LPHO lines 1, 3 and 15 using a rabbit anti-AtHSP101 antibody. Line number is indicated before the decimal point, the number identifying individual T_1 plants follows the decimal point. *Arabidopsis* HSP101 protein was detected in the 1.1, 1.2, 1.3, 3.3, 3.4, 15.1, 15.2, 15.3 and 15.4 extracts but not in the inflorescences or HTS leaf extracts of control *B. napus* DH12075.

Figure 4.4 DH12075 and LPHO plants 42 days after planting.
DH12075 (left) and transgenic LPHO (right) *B. napus* plants were sown on the same day and grown under non-stress conditions. DH12075 plants had fewer leaves flowered before the LPHO plants. HPLO plants planted at the same time and grown in the same growth chamber had similar phenology to the LPHO plants (not shown). Furthermore, both types of transgenic plants produced more leaves than DH12075 plants prior to flowering.

opened an average of 40.4 ± 1.43 days after planting. T_1 HPLO plants (n=141) took an average of 45.0 ± 3.33 days to the first open flower (n=141). The difference between DH12075 and HPLO flowering times was significant ($p \ll 0.001$, $t = -10.52$, n=51) using a pooled Student's T-test with uneven variances (heteroscedastic). The average number of leaves per plant prior to bolting increased from 8 in DH12075 to approximately 11 in the transgenics (Fig. 4.4). Leaf, flower and silique morphology of HPLO and LPHO plants were the same as control plants. To synchronise flowering DH12075 plants were sown 10 days after LPHO and HPLO transgenic plants for the HTS experiments. Flowering date and leaf number appeared to be normal in *B. napus* and *Arabidopsis* plants transformed with the other constructs used in this project.

Ten lines of *B. napus* 35SHSP and two lines of *B. napus* EntCupHSP were produced while ten lines each of *Arabidopsis* 35SHSP and EntCupHSF were formed (Table 4.2). The presence of the transgene was confirmed in these plants by PCR amplification of NPTII from genomic DNA.

4.3.2 35S and EntCup3 *promoter activity in* B. napus *flowers.*

Flowers from 35SGUS and EntCup3GUS *B. napus* plants were stained for GUS activity to determine if the transgene promoters were active in the target tissues (developing microspores and ovules). GUS activity was observed in the sepals, filament of the anther, base of the corolla and style of 35SGUS plants while strong GUS activity was observed in all parts of the flower except the ovary and pedicel of EntCup3GUS plants (Fig. 4.5). No increase in GUS activity was observed in EntCup3GUS flowers collected from plants during the hottest part of the HTS treatment.

4.3.3 Silique production by HTS-treated transgenic plants

Silique production by *B. napus* LPHO and HPLO plants was the same as in untransformed DH12075 plants (Fig. 4.6). Silique production before, during and after a HTS was similar in DH12075 plants and transformed lines (Fig. 4.6). Slightly higher production of siliques in some transformed lines was not observed when used in a repetition of the experiment.

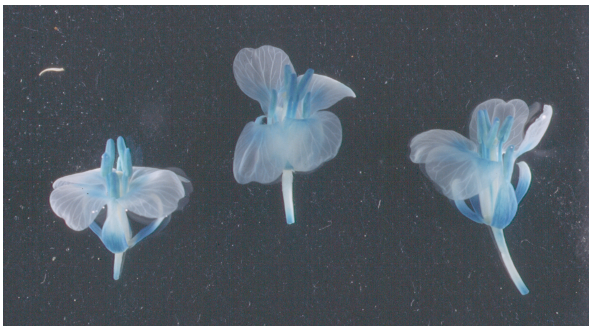


Figure 4.5 *EntCup3* promoter activity in transgenic *B. napus* flowers.
Flowers from *B. napus* plants containing the EntCup3GUS construct were stained for GUS activity which is present at the tip of the wounded pedicle, sepals, anthers, stigma and style. No activity was observed in the petals or ovaries.

Reciprocal crosses were performed between LPHO lines 15, 19, 21 and 22 and HPLO lines 3, 14 and 16 as hybrid plants containing both constructs were expected to have higher levels of AtHSP101 during HTS. AtLFY is a transcription factor that induces greater transcription of its own promoter, therefore, increased production of AtLFY, induced from the HPLO construct by HTS, would be expected to upregulate transcription of the LPHO construct resulting in higher levels of AtHSP101. Plants containing both transgenes were not examined for higher levels of AtHSP101, however.

Confirmation of the presence of both transgenes in hybrid plants was carried out using PCR amplification of the transgenes (Fig. 4.7). A specific LPHO fragment of 545 bp was produced using a 5' *AtLFY* primer and a 3' *AtHSP101* primer (Fig. 4.7). Similarly, a specific HPLO fragment of 439 bp was produced using a 5' *AtHSP101B* primer and a 3' *AtLFY* primer. Thirty-six of the tested progeny contained both DNA constructs while 43 contained only the LPHO construct, 10 only the HPLO construct and 11 plants had neither construct.

All LPHO x HPLO progeny were tested for improved thermotolerance using the standard HTS programme. Since no differences in silique production were observed between the different hybrid lines the data from those plants containing the same transgene(s) were pooled (Fig. 4.8). No improvement in silique production compared to control plants was observed during or after HTS treatment of plants containing a single or both transgenes.

Production of siliques containing seeds by 35SHSP and EntCup4HSP *B. napus* plants was no greater than that of DH12075 control plants under control or HTS conditions (Fig. 4.9). The transgenes did not improve thermotolerance under the HTS conditions used here. No differences were observed in the number of seed-containing silique produced by HTS or control treated 35SHSP or EntCup4HSP plants compared to DH12075 plants (Fig. 4.9).

Untransformed wild-type *Arabidopsis* produced the same weight of seeds under HTS and control conditions. The difference in seed production was not significant between the treatments (t (two-tailed) = -0.67, $df = 5$; $p = 0.56$). The weight of seeds per plant from HTS-treated plants from any line (transformed or untransformed) was not significantly different from the weight of seeds from the non-HTS-treated plants

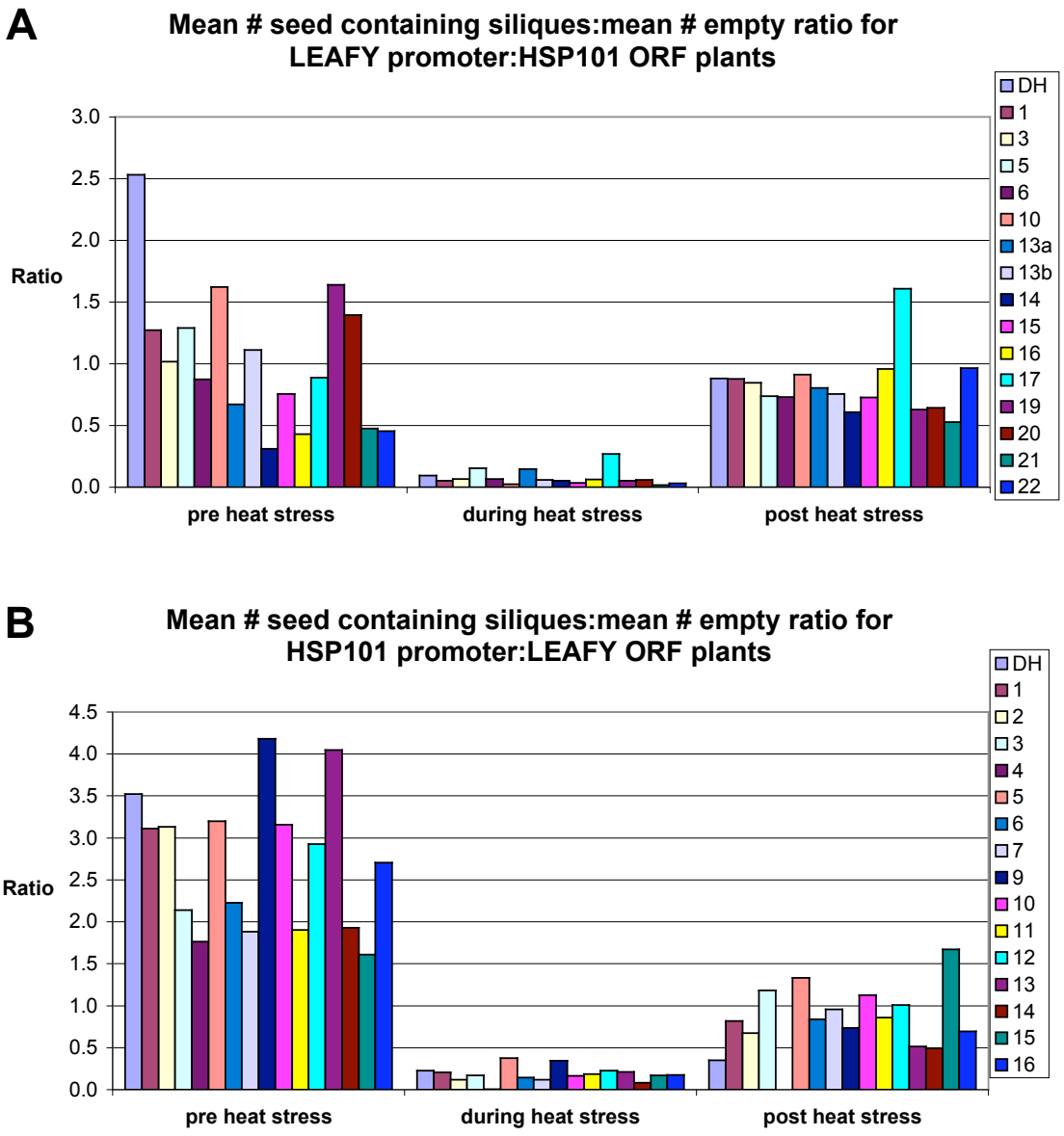


Figure 4.6 Silique production by transgenic HPLO and LPHO *B. napus* plants.

The ratio of seed containing siliques: empty siliques (either aborted pistils or parthenocarpic siliques) by lines of transgenic *B. napus* plants containing the LPHO (A) or HPLO (B) transgene is shown. Each bar represents the average number of seed containing siliques : empty siliques for 10 plants per line before, during and after HTS treatment. The LPHO lines indicated in (B) are the offspring of those shown in Figure 4.2.

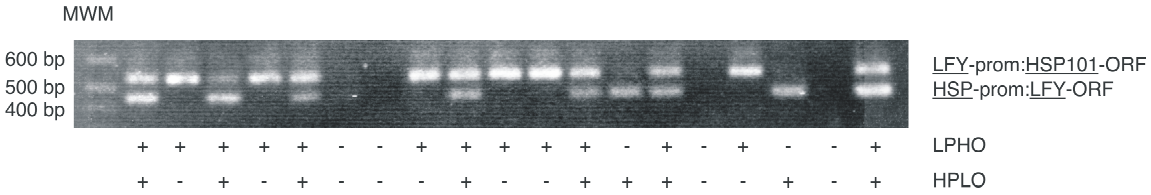


Figure 4.7 PCR analysis of LPHO x HPLO cross progeny.
Fragments of the *Arabidopsis HSP101* and *LFY* genes were amplified by PCR from DNA from LPHO x HPLO progeny. Primers amplified a small fragment of each transgene: 545 bp LPHO fragment and 439 bp HPLO fragment. The presence of each transgene in individual plants is indicated by + and the absence by -. Results from individuals 42-59 (of 100 progeny) are shown.

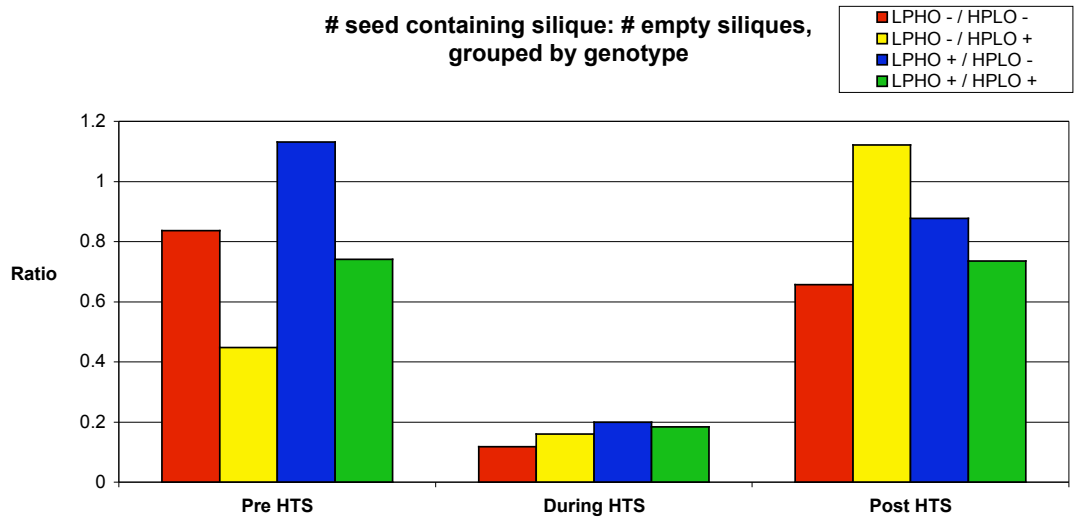


Figure 4.8 Silique production by HPLO x LPHO progeny plants.
Progeny from LPHO x HPLO crosses were HTS-treated for one-week and the number of seed-containing siliques:empty siliques determined. The mean ratio for the different plant populations is shown for each time period of the experiment. Red bars (■) represent control plants (n=11), yellow bars (■) plants with only HPLO (n=10), blue bars (■) plants with only LPHO (n=43) and green bars (■) plants with both HPLO and LPHO (n=33).

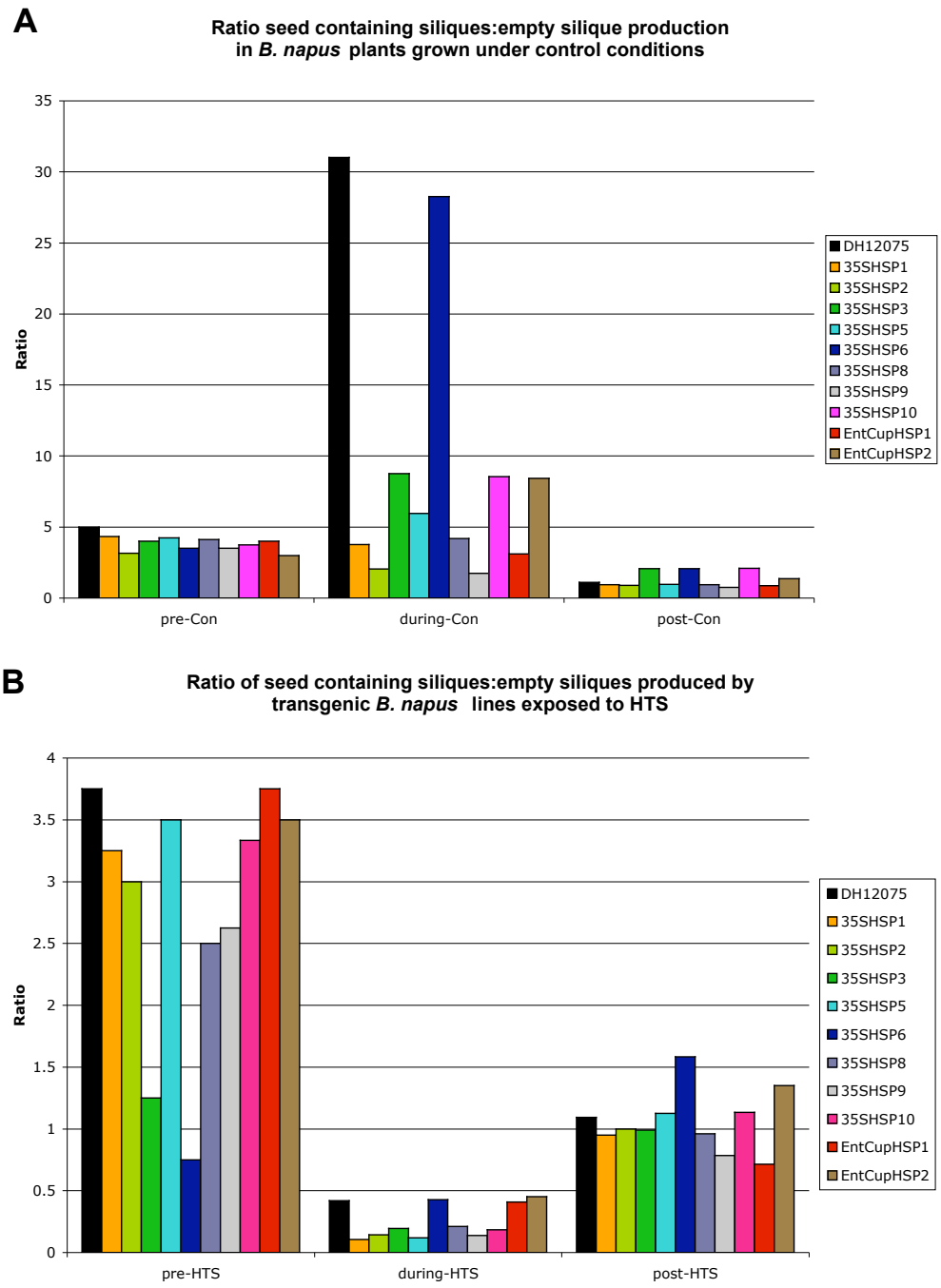


Figure 4.9 Silique production in HTS and Control *B. napus* 35SHSP and EntCupHSP lines.

A) Seed producing silique :empty silique ratio of DH12075 plants, ten 35SHSP lines and two EntCup4HSP lines grown under control conditions. Three time periods corresponding to the HTS treatments are indicated as pre- during- and post-, although all plants remained at control temperatures throughout the experiment. B) The same lines treated with HTS for one week. Silique production during three time periods is indicated (pre-, during- or post-HTS), depending on when the flower that produced a particular silique opened.

of the same line (Table 4.3A). The HTS treatment used did not appear to affect seed production in either the transgenic lines or untransformed control plants.

Seed production by *Arabidopsis* plants containing the EntCup4HSF construct was not significantly different from wild-type Columbia plants (Table 4.3A). There were no significant differences between EntCup4HSF lines and untransformed control plants or between HTS-treated and control temperature-treated plants at a 0.05 significance level.

No significant difference was observed between HTS and control plants containing the 35SHSP construct (Table 4.3B). A significant difference in seed production was observed between 35SHSP lines, however. The differences between transgenic lines of plants was probably due to the fact that four EntCup4HSF lines and three 35SHSP lines produced abnormally low amounts of seed (i.e. 15 mg vs an average of ~115 mg of seed produced by the other plants).

4.4 Discussion

Any strategy designed to overcome the effects of HTS on plant reproduction will need to take a complex variety of factors into account. HTS has multiple effects on the physiology and biochemistry of plant cells and tissues. By overexpressing HSPs, thereby protecting cells from the effects of irreversible protein denaturation, the transgenic plants described in this chapter were designed to overcome reductions in seed production caused by HTS. The hypothesis, that overexpression of HSPs in transgenic plants would improve flower thermotolerance, was reasonable given that overexpression of *HSP101* in *Arabidopsis* improved seedling thermotolerance (Hong et al., 2000; Queitsch et al., 2000) and that *Arabidopsis hsp101* mutants showed reduced seedling thermotolerance (Hong et al., 2001). Therefore, I attempted to improve flower thermotolerance by transforming plants with the LPHO construct, which was expected to target *AtHSP101* expression to early flower development via the *AtLFY* promoter (Busch et al., 1999). Expression of AtHSP101 was detected in the inflorescences of LPHO plants (Fig. 4.3) demonstrating that the transgene was functioning as expected. No improvements in thermotolerance were observed in these transgenic lines, however

Table 4.3 Two way ANOVA comparing seed weight in transgenic *Arabidopsis* lines.

A) Variation in seed weight from control and HTS-treated EntCup4HSF lines was analysed. No significant differences between line or treatments was observed (at 0.05 significance level). B) ANOVA for the 35SHSP lines showing no significant difference between HTS and control plants or between lines.

A

EntCupHSF	Sum of Squares	df	Mean Square	F	p
Line	34576.8	10	3457.7	1.862	0.079
HTS plants	1362.2	1	1362.2	0.733	0.397
Interaction	10773.4	10	1077.3	0.580	0.821
Error	78008.7	42	1857.3		
Total	956840.2	64			

B

35SHSP	Sum of Squares	df	Mean Square	F	p
Line	46496.5	10	4649.7	2.385	0.025
HTS plants	926.6	1	926.6	0.475	0.494
Interaction	12051.7	10	1205.2	0.618	0.790
Error	79946.4	41	1949.9		
Total	883503.0	63			

(Fig. 4.6). In the developmental stages of this project, it was thought that flower abortion during HTS was a major cause of reduced seed set. The HPLO construct was designed to "force" flower development during HTS by overexpressing LFY. LFY induces floral meristem development (Weigel and Meyerowitz, 1993; Wilhelmi and Preuss, 1997); therefore, it was thought that HTS-induced expression of LFY could overcome the purported flower abortion. High levels of HSP101 production during HTS were expected in the progeny plants that contained both constructs from the LPHO x HPLO crosses. It was hypothesized that HTS would induce increased transcription of the HPLO construct and the resulting LFY protein (plus endogenously produced LFY) would induce transcription of *LFY* promoters, thereby increasing the transcription of the LPHO construct. Although 36 LPHO x HPLO progeny had both transgenes no improvements in thermotolerance were observed over progeny containing one or no transgene.

Improvements in thermotolerance were also expected in *B. napus* plants containing the 35SHSP and EntCup4HSP. It was thought that, as in *Arabidopsis* (Hong et al., 2000), constitutive expression of *HSP101* would improve overall thermotolerance, including flower thermotolerance. This was not observed, however. Although the *EntCup4* promoter is reported to be constitutively expressed in flowers (Wu et al., 2001) it is not active in all tissues within the flower (Fig. 4.5). Thus transcription of HSP101 and HSF3 may not have occurred in the gametophytes, which remained unprotected.

Comparisons were planned between plants containing the *35S* promoter or the *EntCup4* promoter transgenes as the former is not active in pollen (Odell et al., 1985). Increased thermotolerance in the EntCup4HSP flowers over 35SHSP flowers would have indicated that improving pollen thermotolerance is necessary to overcome HTS-induced inhibition of seed set. Unfortunately this comparison could not be made due to difficulties with low plant transformation efficiencies.

The *Arabidopsis* 35SHSP and EntCup4HSF lines and in the *B. napus* 35SHSP and EntCup4HSP lines might have had increased vegetative tissue thermotolerance. Increased vegetative thermotolerance might not necessarily result in increased reproductive thermotolerance; however, improved vegetative thermotolerance, while not directly improving flower thermotolerance, might result in increased overall plant fitness

during HTS lead indirectly to improved seed production. Therefore a future goal should be to screen homozygous lines for improved vegetative thermotolerance, keeping in mind that reproductive thermotolerance might improve as a consequence. Vegetative thermotolerance was not examined in this work as this subject was considered beyond the scope of the project.

All HTS experiments were limited to T_1 generation *B. napus* and T_2 generation *Arabidopsis* plants because of time constraints. One quarter of the test population derived from T_0 *B. napus* or T_1 *Arabidopsis* plants carrying a single copy of the transgene would not carry the transgene. If the presence of the transgene improved thermotolerance a noticeable increase in silique production would be expected by the heterozygous or homozygous population expressing the transgene.

It is possible that the transgenes improved flower thermotolerance but that the improvement was not observable under the conditions used in this study. *Arabidopsis* plants overexpressing *HSP101* had an increase in basal thermotolerance of approximately 2°C over control plants (Hong et al., 2001). Therefore, it is possible that the HTS temperatures experienced by the transgenic *B. napus* plants in this study were greater than could be accommodated by the protective capabilities of the transgenes. For example, if the transgene had increased the temperature at which total sterility occurred from 30°C to 32°C, the 35°C temperatures experienced by the plants would still have resulted in total sterility regardless of the presence of the transgene.

The experimental conditions used here were insufficient to cause a decrease in seed production by HTS–treated *Arabidopsis* plants. Further work investigating the range of temperatures adversely affecting seed production in *Arabidopsis* and *B. napus* should be determined, therefore. Based on the observations in Chapter 2, a linear relationship between temperature and fertility is likely as the coefficients of regression were fairly high. Therefore, determination of a "Fertility Loss Temperature" (FLT) at which 50% of gametophyte fertility is lost (FLT_{50}) would be useful to measure basal thermotolerance. Transgenic plants with a FLT_{50} higher than control plants, or with a higher rate of fertility at the wild-type FLT_{50}, would have a higher gametophyte basal thermotolerance than control plants. Fertility could be measured by the number of seeds

produced by HTS pollen x Control pistil crosses or perhaps by *in vitro* pollen germination rates (see Chapter 3).

Increased thermotolerance may not have been observed in transgenic plants because of ineffective spatial and/or temporal expression of the transgene. *LFY* is expressed during floral meristem differentiation (Weigel and Meyerowitz, 1993) and very early in flower determination (Busch et al., 1999). Therefore in LPHO plants HSP101 would not have been produced during gametophyte formation which occurs at a much later stage of flower development. Transgenes controlled by the *35S* promoter appear not to be transcribed in developing microgametophytes (Odell et al., 1985). The *EntCup4* promoter is active in mature pollen although activity appears to occur only during late pollen maturation. Therefore, the transgenic ORFs used in this study may not have been expressed during the HTS sensitive critical phase of microgametophyte development, or not at a sufficiently high level to result in thermotolerance. Neither the *35S* nor the *EntCup4* promoters were active in the ovary so the transgene did not provide thermoprotection to the developing megagametophytes (see Chapter 3 for megagametophyte sensitivity to HTS).

Both LPHO and HPLO plants produced more leaves per plant and flowered later than DH12075 plants (Fig. 4.4). These observations suggest that conversion of the apical meristem into the inflorescence meristem was delayed, possibly due to co-suppression of *B. napus LEAFY* (*BnLFY)* by the transgenic *AtLFY*. Overexpressing *AtLFY* in *Arabidopsis* and aspen (Weigel and Meyerowitz, 1993) and *Eucalyptus globulus LEAFY* in *Arabidopsis* (Southerton et al., 1998) resulted in earlier flowering. This suggests that cosuppression of *BnLFY* activity might have occurred in my transgenic plants as a delay in flowering was observed when a transgenic *AtLFY* was present. Indeed, an inhibition of meristem conversion was observed in *Arabidopsis* plants transformed with a rice *LEAFY* orthologue under control of the CaMV *35S* promoter (Kyozuka et al., 1998). Delaying the conversion of the vegetative meristem to the inflorescence meristem would have allowed for the development of more leaf primordia, thus explaining the additional leaves observed in the transgenic plants.

A constitutive low level of *AtLFY* mRNA would have been present in HPLO lines because the *HSP101B* promoter is constitutively expressed at a low level in my

transgenic plants (see Chapter 5). The transcription of the LPHO construct probably incorporated the *AtLFY* 5'UTR fused onto the *AtHSP101* mRNA. These observations suggest that the presence of *AtLFY* mRNA or the *AtLFY* 5'UTR may have been sufficient for cosuppression of *BnLFY* activity.

Cosuppression of *BnLFY* activity by *AtLFY* mRNA or 5'UTR may have resulted from the high degree of similarity between the two mRNAs (Kyozuka et al., 1998; Rottmann et al., 2000). Constitutive expression of *AtLFY* in transgenic *Arabidopsis* plants did not show cosuppression (D. Weigel, pers. com), nor did constitutive expression of eucalyptus *LFY* in *Arabidopsis* (Southerton et al., 1998). Overexpression of *Arabidopsis LFY* in rice resulted in delayed flowering (Kyozuka et al., 1998) however, but and overexpression of a *Populus LFY* did not induce earlier flowering in transgenic *Arabidopsis* or aspen (Rottmann et al., 2000). One hypothesis is that expression of an orthologous gene from a different species may cause cosuppression. To test this possibility, both *B. napus* and *Arabidopsis* plants should be transformed with only the *LFY* promoter including the 5'UTR used in this project and examined for delayed flowering. Additionally, the *AtLFY* 5'UTR alone under control of the *35S* promoter should be inserted into plants to determine if the 5'UTR alone can produce the delayed flowering phenotype. A difference in resulting *Arabidopsis* and *B. napus* phenotypes would show whether cosuppression of *LFY* was greater when the transgene is an orthologous or homologous gene.

Overexpression of a suite of HSP genes was attempted by transforming *Arabidopsis* with *AtHSF3* under the control of the constitutive *EntCup4* promoter. Overexpression of *AtHSF3* in *A. thaliana* led to an increase in HSP production as well as an increase in seedling thermotolerance (Schoffl et al., 1998). Assuming the expression pattern of the EntCup4HSF construct in *Arabidopsis* flowers to be the same as the expression pattern of EntCup3GUS in *B. napus* flowers, I would have expected to see HSF3 expression in the later stages of *Arabidopsis* pollen development. But expression of HSF3 was insufficient to improve thermotolerance in developing pollen. As mentioned previously, the *EntCup4* promoter was not active in *B. napus* ovaries; therefore I assumed it would not be active in *Arabidopsis* ovaries. Hence, I would not

expect *Arabidopsis* megagametophytes developing during HTS to be protected by the product of the EntCupHSF transgene.

There are three possible reasons why HSF3 might fail to improve pollen thermotolerance. Firstly, there is normally a high level of many developmentally regulated *HSP* transcripts already present in developing microspores (Dupuis and Dumas, 1990; Young et al., 2001). Even if HSF3 was able to further induce *HSP* transcription the additional *HSP* mRNAs might not have improved thermotolerance above that already supplied by the HSPs normally present in developing microspores. Secondly, differential translation of *HSP* mRNA might occur in the developing microspores. Young *et al* (2001) showed that even though *HSP101* mRNA accumulated in developing microspores exposed to HTS, a concomitant increase in HSP101 protein was not observed. Finally, post-translational regulation might prevent HSF3 activity. At control temperatures *Arabidopsis* HSF is bound and held inactive by HSP70, preventing HSF trimerization and activation (Hubel et al., 1995). A developmentally controlled increase in HSP70 concentration in maturing microspores might prevent HSF3 from forming activate trimers. The overexpression of HSF3 in developing pollen is still a good option for improving microgametophyte fertility during HTS, however. In future transgenic plants, expression of HSF should be targeted, with a very strong promoter, to earlier stages of microspore development. Furthermore, to prevent HSP70 inactivation of HSF trimerization, a *HSF* gene from a different species should be used. *Arabidopsis* HSF in tobacco was not bound by endogenous HSP70 and was able to trimerise and induce *HSP* transcription at control temperatures (Schoffl et al., 1998). Confirmation that AtHSF3 induced increased *HSP* transcription was not examined in the EntCupHSF plants, however.

Evaluation should be carried out on individual plant lines rather than on groups of plants from different transgenic lines. Lines displaying vegetative thermotolerance should also be tested for improved reproductive thermotolerance in individual plants. Studying the responses of individual to HTS allow identification of individuals with increased thermotolerance, rather than mixed populations of heterozygous and homozygous plants. To determine increased thermotolerance in populations of plants requires a large amount of space and time, especially if more than one line is examined.

Future evaluations should be carried out on homozygous lines to avoid any variability between heterozygous and homozygous plants.

Modifications of the HTS treatment should also be considered, e.g., controlling humidity and using less extreme temperatures. Although it has been shown that HSP expression improves thermotolerance, the biochemical and/or physiological mechanisms by which HTS reduces gametophyte fertility are not known. Creating transgenic plants containing multiple, putative thermotolerance-inducing genes should be developed, therefore. Improving thermotolerance to extreme conditions, like those in this study, may require a change in several physiological factors, such as the scavenging of reactive oxygen intermediates (Kubo et al., 1999; Liu et al., 2000), changes to fatty acid composition in the membranes (Murakami et al.) and/or modification of photosynthate transport within the plant (Aloni et al., 2001; Triboi and Triboi Blondel, 2002).

Some transformations did not produce transgenic plants. I had planned to compare 35SHSP and EntCup4HSP, 35SHSF and Ent4CupHSF activity in both *B. napus* and *Arabidopsis*. Unfortunately, transformation success using these constructs was variable. All of the constructs, except EntCup4GUS were used to transform both species. I suspect that variations in the *Agrobacterium* concentrations used in the transformations were responsible for the observed variability in transformation rates.

4.5 Conclusions

I attempted to produce transgenic plants with thermotolerant flowers in this study. Although several different constructs were used, none improved flower thermotolerance under my experimental HTS conditions. Further testing of the transgenic plants is required. Producing thermotolerant plants will probably require the pyramiding of multiple transgenes before appreciable improvements in reproductive thermotolerance are observed. Strongly transcribing promoters with expression targeted to the thermosensitive developmental stages of the gametophytic tissues will be required. In the current socio-political environment there is concern that transgenic species with improved tolerance to abiotic stresses could become "superweeds". There is some risk that plants with greater tolerance to abiotic stresses could become problems but the risk is the same whether the plant is transgenic or the result of "traditional"

breeding techniques. Therefore arguments against the release of stress tolerant plants should not be based on the technique used to obtain the plants but on the trait itself.

CHAPTER 5 CHANGES IN THE ACTIVITY OF AN ARABIDOPSIS HSP101 PROMOTER LOCATED IN A NONAUTONOMOUS MUTATOR-LIKE ELEMENT.

5.1 INTRODUCTION

Transposons change gene function thereby generating genetic variation (Bennetzen, 2000). The well described *Mutator* (*Mu*) transposons in maize can cause insertional disruption of genes (Bennetzen, 1996; Bennetzen, 2000) and change gene expression (Greene et al., 1994). In some cases portions of a gene or gene fragments may be incorporated into the transposon itself (Bennetzen, 2000; Yu et al., 2000). The *Mutator*-Like Element (MULE) transposons of *Arabidopsis thaliana* L. (Le et al., 2000) have been shown to incorporate fragments of genes, usually a continuous section of the promoter, 5' UTR and first exon (Yu et al., 2000). The gene fragments incorporated into these atypical MULEs replace the transposase ORF, thus making these elements non-autonomous.

It is not known if the portions of a captured gene incorporated into a MULE retain their transcriptional activity or if the Terminal Inverted Repeats (TIRs) act as promoters, as they do in the *MuDR* elements, for the captured gene fragment (Raizada et al., 2001). I investigated the transcriptional activity of a MULE transposon in *Arabidopsis* containing a fragment of *HSP101* promoter and ORF (Yu et al., 2000 and supplementary information at http://www.tebureau.mcgill.ca/clonebase/genetics00paper/athsp.html). The 573 bp *HSP101B* fragment has a high degree of similarity to *HSP101* (At1g74310, Hong et al., 2001). A continuous region of the *HSP101* promoter, 5'UTR and 181 bp of the ORF is present between the TIRs of the *MULE*. This arrangement was first identified by Yu et al (2000) but transcriptional activity was not determined. We have named the copy of

HSP101 (promoter, 5'UTR and ORF) incorporated into the *MULE,* along with the 1657 bp immediately 5' of the inserted gene (including the 5'TIR of the MULE), *HSP101B* throughout this chapter. The 2046 bp section of DNA 5' of the *HSP101B* ORF has been called the *HSP101B* "promoter".

In *Zea mays*, the TIR of the *MuDR* transposon acts as the promoter for transcription of the *Mu* transposase (*mudrA*) allowing regulation of transposition of both the *MuDR* transposon as well as any other non-autonomous *Mu* transposons (Hershberger et al., 1995; Raizada et al., 2001). It is not known if the TIRs of the *MULE* family in *Arabidopsis* act in a similar manner. If the *MULE* TIRs act as promoters, transcription of captured gene fragments is possible. Regulation of transcription and transposition of *MULEs* may depend on the methylation state of the DNA (Singer et al., 2001). If the regulation of *MULE* transposition in *Arabidopsis* is similar to that of *Mu* transposons in *Z. mays* then regulatory genes controlling *MULE* methylation, such as *MODIFIER OF PARAMUTATION1*, would affect transcription and transposition of these elements (Lisch et al., 2002).

During stress, mobile elements may act as a source of phenotypic variation arising from gene rearrangements (Capy et al., 2000), especially in individuals carrying mutations in DNA methylation (Lisch et al., 2002; Singer et al., 2001) or *HSP90* function (Queitsch et al., 2002). With the aid of an autonomous transposase, non-autonomous transposons, such as *MULE-HSP101B*, could conceivably transpose to other sections of the genome during stress or following DNA demethylation (Ludwig Muller et al., 2000).

In plants, *HSP101* is necessary for thermotolerance (Hong et al., 2000; Hong et al., 2001; Nieto-Sotelo et al., 2002; Queitsch et al., 2000). Three copies of *HSP101* have been reported in the *Arabidopsis* genome at gene loci At1g74310, At4g14670 and At5g57710. During heat stress, heat shock transcription factors (HSF or HSTF) bind to heat shock elements (HSEs) of Heat Shock Protein (HSP) promoters to induce transcription (Hong et al., 2001; Nieto Sotelo et al., 1999; Schoffl et al., 1998). At least five putative HSEs have been identified in the regulatory region of *HSP101* (At1g74310), although only four were needed for high temperature stress (HTS) induced transcription (Hong et al., 2001).

Low temperature inducibility requires the presence of low temperature responsive elements (LTREs) such as those observed in the *Arabidopsis COLD REGULATED 15a* (*COR15a*) promoter (Baker et al., 1994). Transcription of *COR15a* is induced by LTS treatment (2°C for 24 hr) in leaves, apical meristems and stems. The *COR15a* LTREs also control a developmental pattern of expression inducing transcription in the anthers of plants grown at control temperatures. GUS activity in transgenic *COR15a:GUS* plants was observed in all flower organs except ovaries and ovules in LTS treated plants (Baker et al., 1994).

Here I report that the truncated *HSP101* promoter in *MULE-24:HSP101,* which contains just one complete and one partial HSE, was sufficient to induce transcription under heat stress conditions in leaves and flower buds. Furthermore, activity of the *HSP101B* promoter was observed in stably transformed *Brassica napus* and *Linum usitatissimum* and transiently transformed *Arabidopsis* exposed to low temperatures possibly due to the presence of two pairs of LTREs upstream of *MULE-24:HSP101*. I speculate that transcription of endogenous *HSP101B* is regulated by DNA methylation in wild-type *Arabidopsis*. In transgenic *Arabidopsis*, *B. napus* and *L. usitatissimum* plants the unmethylated *HSP101B* promoter regulates expression differently from the endogenous *Arabidopsis HSP101B.*

5.2 Materials and Methods

5.2.1 Cloning and analysis of HSP101B

A 2046 bp fragment upstream of the *HSP101B* start codon was amplified from the *Arabidopsis thaliana* eco. Columbia BAC F3D13 (Genbank acc. #AC004218) using specific primers (5'-GCGGGATCCTGTAGAGTTGATACGAAGTTG-3' and 5'-GGCCTGCAGCTTCGATTAGCCTTTTAAAATCC-3'). The fragment was ligated into pBluescriptKS+ and sequenced in both directions at the DNA sequencing facility, PBI / NRC, Saskatoon. Comparisons between the Chromosome IV *HSP101B* and the Chromosome I *HSP101* (At1g74310) were performed using the ClustalW program at the National Research Council of Canada/Canadian Bioinformatics Resource website (http://www.cbr.nrc.ca/index_e.php) and the BLAST program at NCBI (Altschul et al., 1990). Identification of putative promoter motifs was performed using the PLant Cis-

Acting regulatory Element (PLACE) database (http://www.dna.affrc.go.jp/htdocs/PLACE/).

5.2.2 Induction and detection of HSP101 *and* HSP101B *transcripts in* Arabidopsis

Pots of *Arabidopsis thaliana* eco. Columbia plants were grown in a growth chamber with 16 h / 8 h, 23° / 18°C, day / night cycles. Several plants were grown in each 250 mL pot. Pots of mature, flowering *Arabidopsis* plants were either heat stressed at 35°C for one hour, cold stressed at 4°C for four hours or left at 23°C prior to tissue collection. Leaf tissue and unopened flower buds were collected and total RNA extracted using the TRIzol reagent (InVitrogen Canada, Burlington, ON). One Step RT-PCR amplifications (QIAgen, Mississauga, ON, Canada) were performed using 320 ng or 640 ng of total RNA for detection of *HSP101B* and *HSP101* transcripts, respectively. Specific amplification of *HSP101* or *HSP101B* used 3' primers specific for *HSP101* (5'-GCCCATATCAGATTAGGT -3') or *HSP101B* (5'-TTGATCACTCTTTCAGCA -3') along with the same 5'primer (5'-AATCGAAGATGAATCCAG-3') in both reactions. The RT-PCR products were predicted to be 213 bp and 209 bp for *HSP101* and *HSP101B,* respectively. Reverse transcriptase reaction temperature was at 45°C and a 50°C annealing temperature was used in the 25 cycles of the PCR amplifications for both *HSP101* and *HSP101B*. Primers to amplify 18S rRNA and identical competing primers (competimers with a terminal dideoxynucleotide) were included in each reaction as an internal control (Sung et al., 2001). *HSP101* or *HSP101B* RT-PCR products were cleaned using the QIAquick PCR purification kit (QIAgen, Mississauga, ON) and sequenced from the 5' primer (DNA services, PBI / NRC, Saskatoon).

5.2.3 Transformation of flax and canola with the HSP101B:GUS *construct*

The 2046 bp of *HSP101B* was excised from pBluescriptKS+, ligated upstream of the *GUS* ORF in pCAMBIA1381Z (CAMBIA, Canberra, Australia) and used to transform *Agrobacterium tumefaciens* LBA4404. The transformed *Agrobacterium* was used to stably transform *Linum usitatissimum* {flax, \Pylatuik, 2003 #152} and *Brassica napus* (canola, Moloney et al., 1989) and to transiently transform *Arabidopsis* seedlings (McIntosh et al., unpublished).

T_1 *L. usitatissimum* and *B. napus* seeds containing the *HSP101B:GUS* construct were imbibed in Petri dishes on moist filter paper. After 7-10 days, emergent seedlings were placed in microcentrifuge tubes containing 500 µl of sterile distilled water, which were incubated in the light at various temperatures for 30 (canola) or 40 (flax) minutes. The water was removed and seedlings fixed with ice-cold 90% acetone for 15 minutes. Histochemical staining of tissues from transformed plants was performed using the method of Sieburth and Meyerowtiz (1997).

Flowers were collected from control and HTS-treated *L. usitatissimum* and *B. napus* T_1 plants grown in growth chambers with 16 h / 8 h, 23°(25° for flax) / 18°C, day / night cycles. Flowers were fixed and stained as above. Since the *HSP101B:GUS* construct would segregate in T_1 plants, non-staining seedlings or flowers were disregarded. Multiple flowers from each line and each treatment were stained to determine GUS activity in flowers containing the construct.

5.2.4 Determining methylation of HSP101B *using PCR*

A. thaliana eco. Columbia genomic DNA (500 ng) was digested with an excess of HpaII or MspI for 5 hours. Both restriction enzymes recognize and cut 5'CCGG3' sequences but HpaII is sensitive to methylation of either cytosine while MspI is sensitive to methylation only of the first (Jeddeloh and Richards, 1996; Sneider, 1980). The DNA was cleaned using phenol:chloroform:isoamyl alcohol (25:24:1), ethanol precipitated and resuspended in the original volume of sterile water. PCR amplifications using primers flanking the HpaII/MspI recognition sites were used to amplify regions of *HSP101B,* using 50 ng of digested, HpaII-digested or MspI-digested genomic DNA as a template. A 10 µL aliquot of the PCR product was separated using a 1.3% agarose gel, viewed and photographed using a BioRad GelDoc system (BioRad).

5.3 RESULTS

5.3.1 Description of the HSP101B promoter region

The *HSP101* fragment incorporated into *MULE-24* consists of 381 bp of promoter and 5'UTR and 192 bp of ORF for a total length of 573 bp (Fig. 5.1A). A high degree of similarity between the *HSP101B* and *HSP101* (At1g74310) sequences has been

maintained, with 501 of 573 bp identical (87.4%) (Fig. 5.1B). Over the comparative length, *HSP101B* is missing 16 bp but includes a 6 bp insert. *HSP101B* had a higher level of similarity to *HSP101* (At1g74310) than to the other two *HSP101* genes (At4g14670 and At5g57710) identified in the *Arabidopsis* genome. Excluding the last 3' 10 bp of *HSP101B* the overall identity increased to 88.8% (500 of 563 bp identical). Sequence identity was higher within the 192 bp of the ORF than in the region 5' to it, with 182 bp (94.8%) identical and zero gaps in the alignment. The calculated amino acid (aa) sequence was 95.2% identical (60 of 63 aa) although there was more divergence towards the amino-terminal end of the predicted peptide sequence. This protein domain is conserved between species, although not as much as the nucleotide binding domains (Agarwal et al., 2002). The intron in the At1g74310 *HSP101* 5'UTR (Fig. 5.1A, as identified in Hong et al., 2001) was also present in the *HSP101B* 5'UTR. The level of identity within the intron (69%, 67 of 97 bp identical) was lower than that in the remainder of the 5'UTR outside the intron (87%, 216 of 248 bp identical), however.

The *HSP101B* sequence is bracketed by *MULE-24* TIRs (Fig. 5.1B, Yu et al., 2000 and www.tebureae.mcgill.ca/clonebase/genetics00paper/athsp.html). The *MULE:HSP101B* is a nonautonomous element lacking a transposase ORF.

Two HSEs are present in the *HSP101B* promoter, one identical to a HSE in the *HSP101* promoter identified by Hong and Vierling (2001) and one with a single base pair difference (Fig. 5.1C, uppercase underlined). Approximately 360 bp upstream of the 5' Target Site Duplication (Fig. 5.1C) are two LTREs in the opposite (antisense) orientation separated by ~350 bp, (Fig. 5.1C, uppercase bold). A further 58 bp upstream of the LTREs on the template strand are two additional LTREs in the sense orientation separated by ~300 bp.

The closest putative genes 5' and 3' to *HSP101B* have no known function (At4g01430 and At4g01440). The ORFs for these two genes both are oriented in the same direction as *HSP101B* and are situated approximately 4 kb on either side of the transposon.

A

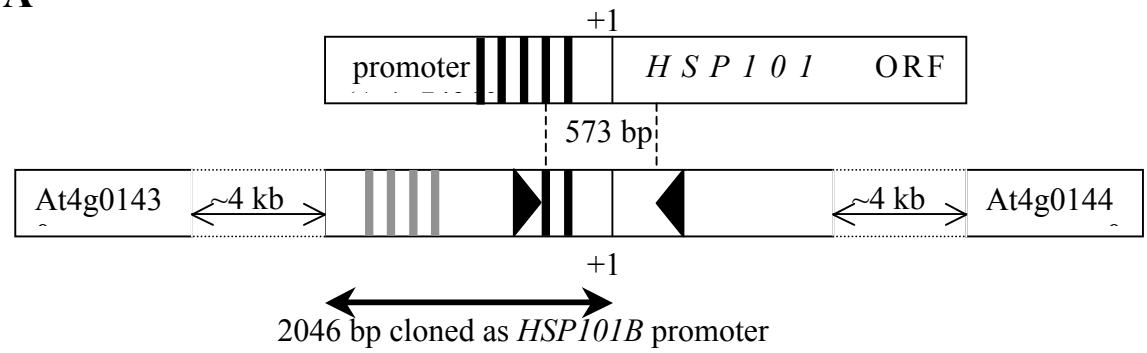


Figure 5.1 Sequence of the HSP101B DNA fragment.
A) Alignment between *HSP101* and *HSP101B*. HSEs are shown as vertical black lines, LTREs as vertical grey lines. The *MULE* TIRs are shown as triangles and bracket the 573 bp of captured *HSP101*. B) alignment of *HSP101B* and *HSP101* (At1g74310) nucleotide and amino acid sequences. Identical nucleotides are indicated with asterixes. The primer sequences used in the RT-PCR to amplify *HSP101B* transcripts specifically are underlined. Start and stop codons and intron are in **BOLD**. Lower case letters indicate sequence not used in the alignment, but included to indicate primer sites. Single letter amino acid sequence for HSP101 is shown with the HSP101B substitutions underneath. C) Sequence of the *HSP101B* promoter cloned from the BAC F3D13. Sequence with high identity to *HSP101* is underlined with start and stop codons **bold underlined**. The two HSEs (one conserved and one with a single nucleotide difference to *HSP101*) and the TATA box are in UNDERLINED UPPERCASE letters. The LTREs are in **UPPERCASE BOLD**. The TIR regions of *MULE-24* are in *italics* while the target site duplications are ***bold italics***. The first 2046 bp (up to the start codon) was cloned and used to regulate GUS expression in transgenic flax and canola

B *HSP101* and *HSP101B* alignment

```
HSP101        GCTTCTAGTTCTATGCAAAAAAACGACGATAGTTCTCTATCTTTCCAGATGAATCTCCTTCCATATACAAAAGCAGTCAT  80
HSP101B       GCTTCTTATTCTATGAAAACAAACGACGATAGTACTCTATCTTTCCAGATGAATCTCCTTCCCTATACAAAAGCC-TCAT  79
              ******  ******* *** ************** ****************************** ***********  ****

HSP101        GCCTCCTCGCTCTCTCGCAATTCACAAAGTATCCAAACATCTAAAGTTATCAATTTTACAACATTACCGCTATAATCTGC 160
HSP101B       GCCTCCTCGCTCTCTCGCAATTCACAAAGTTTCCAAACATCTAAAGTTTTCAATTTTACAATATTATCGCTATCATCTGC 159
              ******************************** ***************** ************ **** ****** ******

HSP101        TTGATTCTCTGCAAAAAGAGAAGACTTTTTACCGAGAAGAAG------TCCTCTGGCTCATTGAAGAAACTCAACGAAAC 234
HSP101B       TTGATTCTCTGCAAAAAGAGAAGACTTTA-ACTGAGAAGAAGAAGAAGTCGTCTGATTTATTGAAGAAACTCTGCGAAAC 238
              ***************************  ** *********      ** ****  * *************  ******

HSP101        AAACCCAGTTCTCATATATCGTTTTAAG[GTAAATGATCGCGACAATCTTGTTCTCATTTGTGTGTTTTTGTGTTTGTGAT] 314
HSP101B       AAACTCAGTTCTCATA--TCGTTT-AAG[GTAAATAAGCGCAACATTCTTTTTTCAATTTGTGTGTTTGT-------CGAT] 308
              **** ************  ****** ********* * *** *** **** **   ************ *        ***

HSP101        [TAGGGTTTACAAAAGATACTGAGATTAGTTTTTTTTTTTTTTTTTTTTTTTCAG]GGATTACAAAAG-CTAATCGAAG[ATG] 393
HSP101B       [T---GTATATGAAAGATGCTGAGATTAGGGTTTGTTTGTGTGTTTTTTT--CAG]GGATTTTAAAAGGCTAATCGAAG[ATG] 383
              *   ** **  ****** **********  *** *** * * *******  ********  ***** *************
                                                                                        M
                                                                                        M

HSP101        AATCCAGAGAAATTCACACACAAGACAAACGAGACAATTGCTACAGCTCATGAGCTAGCTGTGAATGCAGGACATGCTCA 473
HSP101B       AATCCAGAAAAGTTCATACACAAAACAAACGAGACAATTGCTACAGCTCATGAGTTAGCTGTGAATGCAGGACATGCTCA 463
              ******** ** **** ****** ****************************** ************************
               N  P  E  K  F  T  H  K  T  N  E  T  I  A  T  A  H  E  L  A  V  N  A  G  H  A  Q
                             I

HSP101        ATTCACTCCTTTGCATTTAGCTGGTGCTTTGATCTCTGATCCCACCGGTATATTTCCTCAAGCAATCTCTAGTGCCGGTG 553
HSP101B       ATTCACTCCTTTGCATTTAGCTGGTGCTTTAATCTCTGACCCCACCGGTATATTCCTTCAAGCAATCTCTAGTGCCGATA 543
              ****************************** ******** ************** * ******************** *
                F  T  P  L  H  L  A  G  A  L  I  S  D  P  T  G  I  F  P  Q  A  I  S  S  A  G
                                                                                    D

HSP101        GCGAGAACGCAGCTCAATCTGCTGAAAGAgtgatcaa
HSP101B       GCGAGAACGCAACTCAATCGAAAACC[TAA]tctgatatgggc
              *********** *******         *
              G  E  N  A  A  Q  S  A  E  R  I
              S           T        K  T  *
```

C *HSP101B* sequence (promoter, 5'TIR, 5'UTR, intron, ORF)

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tgtagagttgatacgaagttgttttgagtggcatttttcgttaatacttatgttggagactatgaattgttatgaaatttt   80
taccattgtctttttttatataattatattttaaccattagtcatattaattagttaaaacgttaacttatttatttatg  160
tatgatttatttttttaattattaaaaatatgaccaataaattgaaatttcttctctctagcgagataaaacattacgtag  240
cgatgtttcaaactaaatttggcttcattacatcttacaattCCGACattttttatgtcttctagttgaacgaaaatattg  320
atgtgtcaggtttgagagacatgggttttagtttaattgtattgatgattttcaaataaatattaatttaagggaaaatt  400
ggaaaaaaagatatctacaaaaaaatttgataactgaaatctattaaaagtatttgttatttttattgctaagatactcag  480
agtatcttaaccaatggagatgtccttagtgttttaattacaatttacataatatgatatatactaattaccccaaataa  560
tcttgtatatatcactcgtCCGACaaacacattgctgagacaggtttttatgtcattaaccaaacattattcaactggtt  640
gaGTCGGttaataaaactcaaggtgcagttggtttataaaactcaagtttacgatcctacttaataatgttattttggta  720
aattatgttgtcattataaaatctagttatgtcgtttaacaagaaaaagtattatttatgtcgtttctaacttttcattt  800
acaattttgtttctttacgtttttctttgccattgatttgatcatgttggcttttatcttcttttggaaaagcaaaggaa  880
agttaaagtcatataaagctttttatttctttcaagtaaaattatagctaGTCGGccaatctgattttcttcgaaagttaa  960
tgggcttattaatatgtgcagcactaaatgatagcacaatttgcatataaaatacatatgccttatgagataaacgaagt 1040
ggcactggcatgacttcggtcgtataccggattcaacaagacacttaaacatctcgacagtgatcgttcggagactatca 1120
tgtccattttatcaaattgacttgtaaaaataagagcaaatgcattttgtgatataaaataatcaaatcaatatttataa 1200
ctcttttcatacactatatctttttttttttttcaaaaataaaaatagtacatgtgttgtatttgaaaaagataatacata 1280
ctttgtagatcc***attatattat***gggaaaaaacctgaaaaaacctccatttatttttaatttgtccgtttaatacatgcgt 1360
*tatttaatttgtctgtttaatacttaagttaatttttatgtcctttaaaaactttcattttctaaaatttttgacaaaatc* 1440
*agacgtctaaaataacgaccgttaacagtggtaacggaatcgctaaccacaattaaaatttaaccctataatgtttacta* 1520
*tttatatacaattgattttctaattgttacgaatttattatcttagtctagtggttatgtgagtatttattattcgaatc* 1600
*accccgagatcaaatctcatgtccaaccaattctttggatttttttccaggatttttttttccctttgcttcttattctat* 1680
gaaaacaaacgacGATAGTACtctatctttccagatGAATCTCCttcccTATAcaaaagcctcatgcctcctcgctctct 1760
cgcaattcacaaagtttccaaacatctaaagttttcaattttacaatattatcgctatcatctgcttgattctctgcaaa 1840
aagagaagactttaactgagaagaagaagaagtcgtctgatttattgaagaaactctgcgaaacaaactcagttctcata 1920
tcgtttaaggtaaataagcgcaacattctttttttcaatttgtgtgtttgtcgattgtatatgaaagatgctgagattagg 2000
gtttgtttgtgtgtttttttcagggattttaaaaggctaatcgaag**atg**aatccagaaaagttcatacacaaaacaaacg 2080
agacaattgctacagctcatgagttagctgtgaatgcaggacatgctcaattcactcctttgcatttagctggtgcttta 2160
atctctgaccccaccggtatattccttcaagcaatctctagtgccgatagcgagaacgcaactcaatcgaaaacc**taa**tc 2240
tgatatgggccgccgtcaggtttaaaaaaaaaaaagaccaaaatattggaggggtgtggtgatcgaactcatgttcacga 2320
*gaataataaaatctcacataaccaatggactaggataatatgttcgtaatgactagaaaaacaattgaatacaagtaaac* 2400
*aaatattatagggttaaatttttaactgtggttagcgatttcgttaccactgttaacggccgttatcttggacgtctgatt* 2480
*ttgccaaaattttaaaaaatgaaagtttttaaaggacataaaattaacttaagtattaaacagacaaattaaataacgca* 2560
*gatattaaacggacaaattgaaaataaatggaggttttttcgggttttttttcc**atcttcttat***tatataaagtacagttt 2640
```

5.3.2 The HSP101B promoter is transcriptionally active in Arabidopsis

Transcription of the *HSP101B* ORF occurred in HTS-treated leaves and unopened flower buds (Fig. 5.2) but *HSP101B* mRNA was not detected in control or LTS-treated tissues. *HSP101* mRNA was detected in unopened flower buds under all conditions and in HTS leaves. Levels of *HSP101* mRNA were elevated in all HTS-treated tissues. These results concur with other findings that, in plants, *HSP101* mRNA is constitutively expressed at low levels and increases during HTS (Hong et al., 2000; Queitsch et al., 2000).

Amplification of the 18S rRNA in HTS-treated leaves and buds was atypical probably because amplification of the 18S rRNA was out-competed by the high amounts of *HSP101* mRNA (Fig. 5.2). The average density of the 18S rRNA bands in LTS treated and control leaves and buds was determined so as to obtain a figure for the amount of 18S rRNA we might expect to observe in these reactions. This figure was used to determine the approximate ratio of *HSP101* mRNA to 18S rRNA. Sequencing of the RT-PCR products confirmed the identities of both the *HSP101* and *HSP101B* fragments.

5.3.3 HSP101B *promoter activity is conserved in flax and canola*

A range of *HSP101B* promoter activity was observed in stably transformed *L. usitatissimum* and *B. napus* seedlings (Fig. 5.3A, B). The activity of the promoter varied between transgenic lines with both constitutive and temperature induced GUS expression observed. In general, a low level of constitutive GUS activity was observed in *L. usitatissimum* seedlings at 25°C although high level of constitutive GUS expression was observed in some lines. The level of GUS activity increased in some lines when LTS (4° or 14°C) or HTS-treated (31° or 35°C) but in other lines the expression levels were considerably higher (Fig. 3A). In *B. napus* three patterns of *HSP101B* promoter activity were observed (Fig. 3B): constitutive, inducted by both HTS and LTS, or induced by LTS alone. In transiently transformed *Arabidopsis*, increased GUS activity was also induced by LTS (4°C) or HTS (35°C) treatment (McIntosh et al., unpublished).

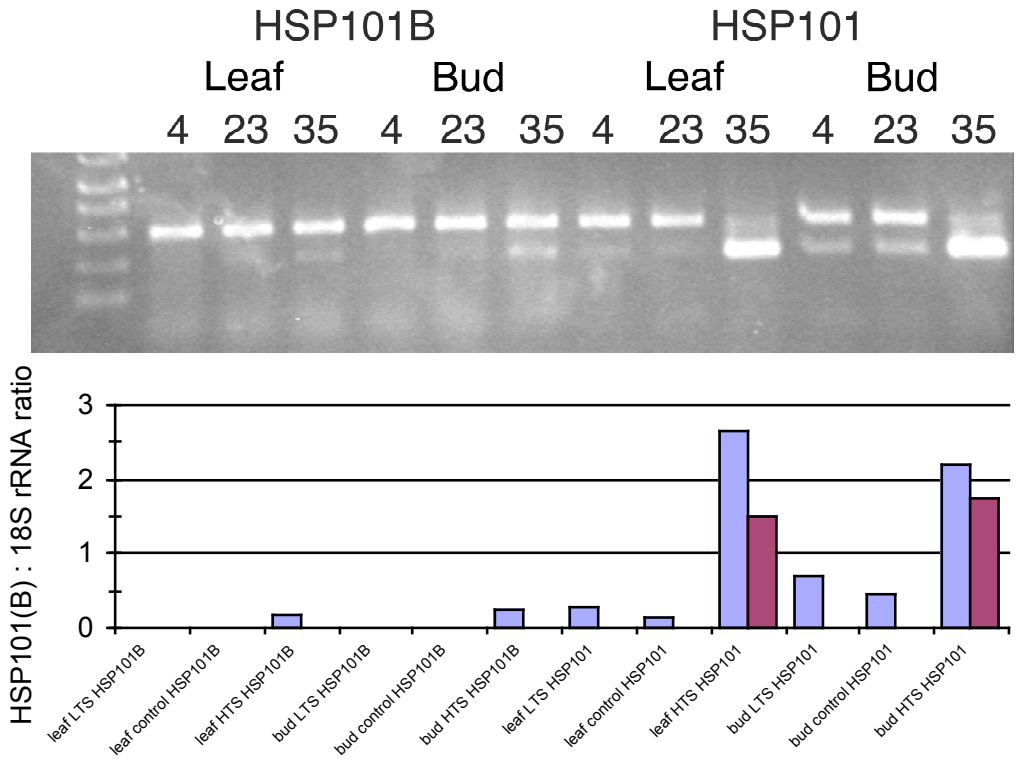
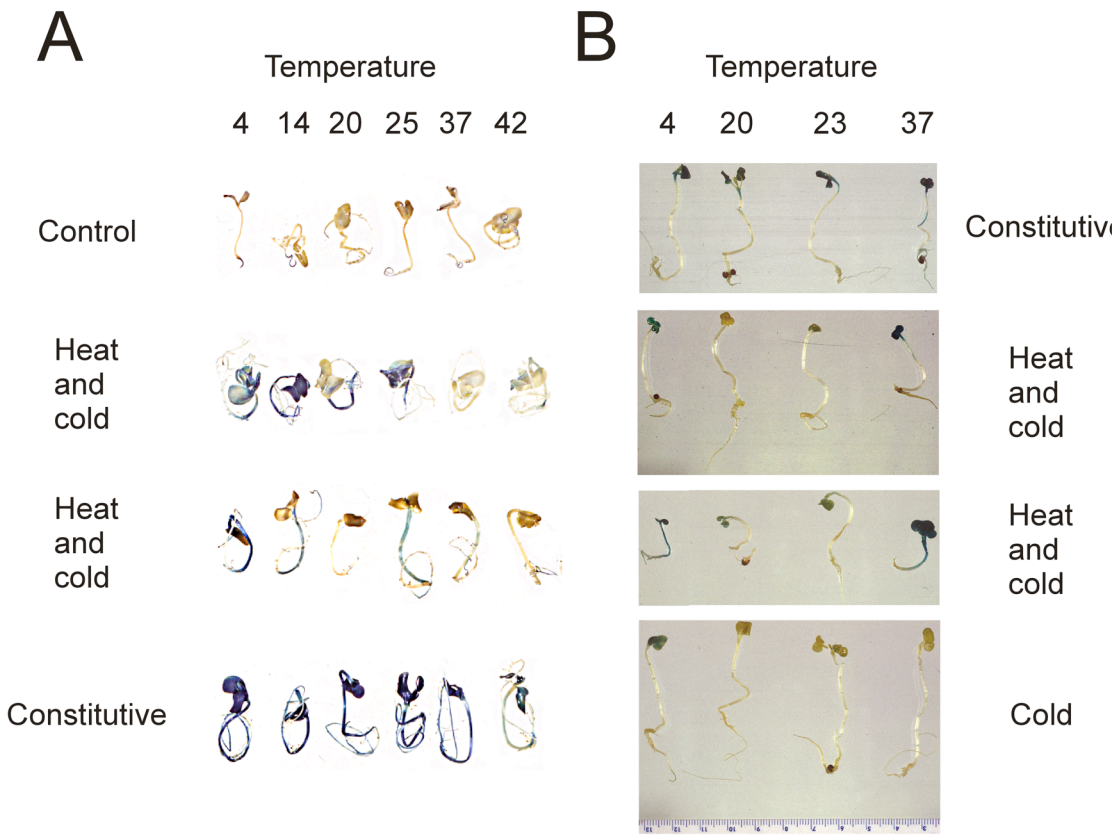


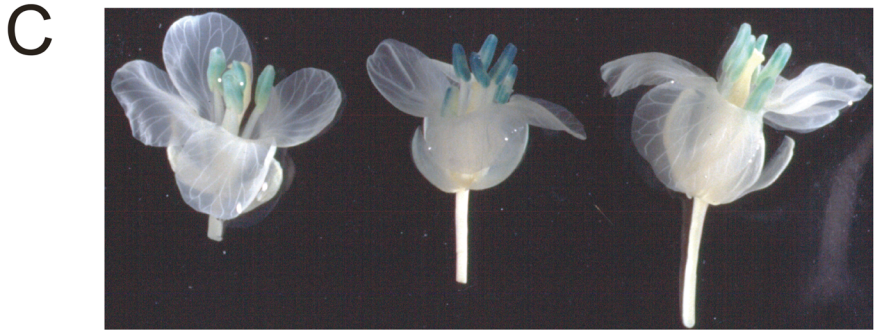
Figure 5.2 *HSP101B* and *HSP101* mRNA transcripts in LTS, control and HTS treated *Arabidopsis*

The presence of *HSP101B* and *HSP101* mRNAs were determined in the leaves and unopened buds of LTS, HTS or control treated *Arabidopsis* plants using RT-PCR. As an internal control, *18S* rRNA was amplified using a 2:8 primer : competimer ratio. The ratio of *HSP101*/*HSP101B* is shown in the graph (blue bars). Purple bars show the *HSP101*: *18S* rRNA ratio using the mean 18S RT-PCR product intensities from the other treatments as amplification of *18S* rRNA was inhibited by competition with HSP101 mRNA in HTS tissues.

Figure 5.3 GUS activity in transgenic flax and canola containing the *AtHSP101B*-promoter:*GUS*-ORF construct.

A) Control and transgenic T_1 flax seedlings treated at different temperatures and stained for GUS. No staining is observed in the control (untransformed) plants. Two patterns of *HSP101B* promoter activity were seen in the transgenic plants: constitutive expression and heat and cold inducible expression. Lack of staining in one of the heat and cold inducible seedlings at 37°C was due to segregation of the construct from this plant. B) *HSP101B* promoter activity in transgenic T_1 *B. napus* plants. Three patterns of expression are observed: constitutive, heat and cold-induced, and cold-induced. C) *HSP101B* activity in transgenic *B. napus* flowers. Flowers from plants grown at control conditions show GUS activity in the anthers. GUS staining in the sepals was also observed; however, it is obscured in this figure.


A
Temperature
4 14 20 25 37 42
Control
Heat and cold
Heat and cold
Constitutive
B
Temperature
4 20 23 37
Constitutiv
Heat and cold
Heat and cold
Cold


C

The *HSP101B* promoter was also developmentally regulated in the flowers of both *B. napus* (Fig. 3C) and *L. usitatissimum* (data not shown). GUS activity was observed in mature pollen from both flax and canola plants. In transgenic canola, only adaxial tips of sepals had GUS activity whereas in transgenic *L. usitatissimum,* sepals, petals and pistils showed GUS activity (see Cross, 2002).

5.3.4 Methylation of HSP101B.

Methylation of the *HSP101B* sequences in genomic DNA was determined by digesting genomic DNA with the mCC and CmC sensitive HpaII or mCC sensitive MspI restriction enzymes (both of which recognize and digest 5'CCGG3'). PCR amplification of the genomic DNA, using primers bracketing the 5'CCGG3' site, would only occur if the DNA is not digested by the restriction enzyme. That is, if the restriction enzyme recognition site was unmethylated then production of a DNA fragment by PCR could only occur from undigested genomic DNA (as both HpaII and MspI would digest the DNA, preventing amplification of the region). If the recognition site was methylated at the first cytosine (mCC), neither HpaII nor MspI would digest the recognition site, enabling PCR amplification of the fragment. Finally, if only the second cytosine was methylated, only MspI would be able to digest the DNA, resulting in PCR products from the undigested and HpaII digested template DNAs.

PCR amplification of genomic DNA digested with an excess of HpaII and MspI indicated that the DNA within *HSP101B* is not methylated outside of the *MULE* as a PCR product was only observed in undigested genomic DNA (Fig. 5.4). The 5'CCGG3' sites within the *MULE* were methylated however, as PCR products were observed in undigested, HpaII- and MspI- digested DNA.

5.4 DISCUSSION

The high level of conservation between the sequences of *HSP101B* and *HSP101*, especially in the ORF, suggests that either the transposition event inserting the MULE into Chromosome IV was relatively recent or that *HSP101B* has a regulatory role in *HSP101B* expression in *Arabidopsis*. If the transposition was a recent event then

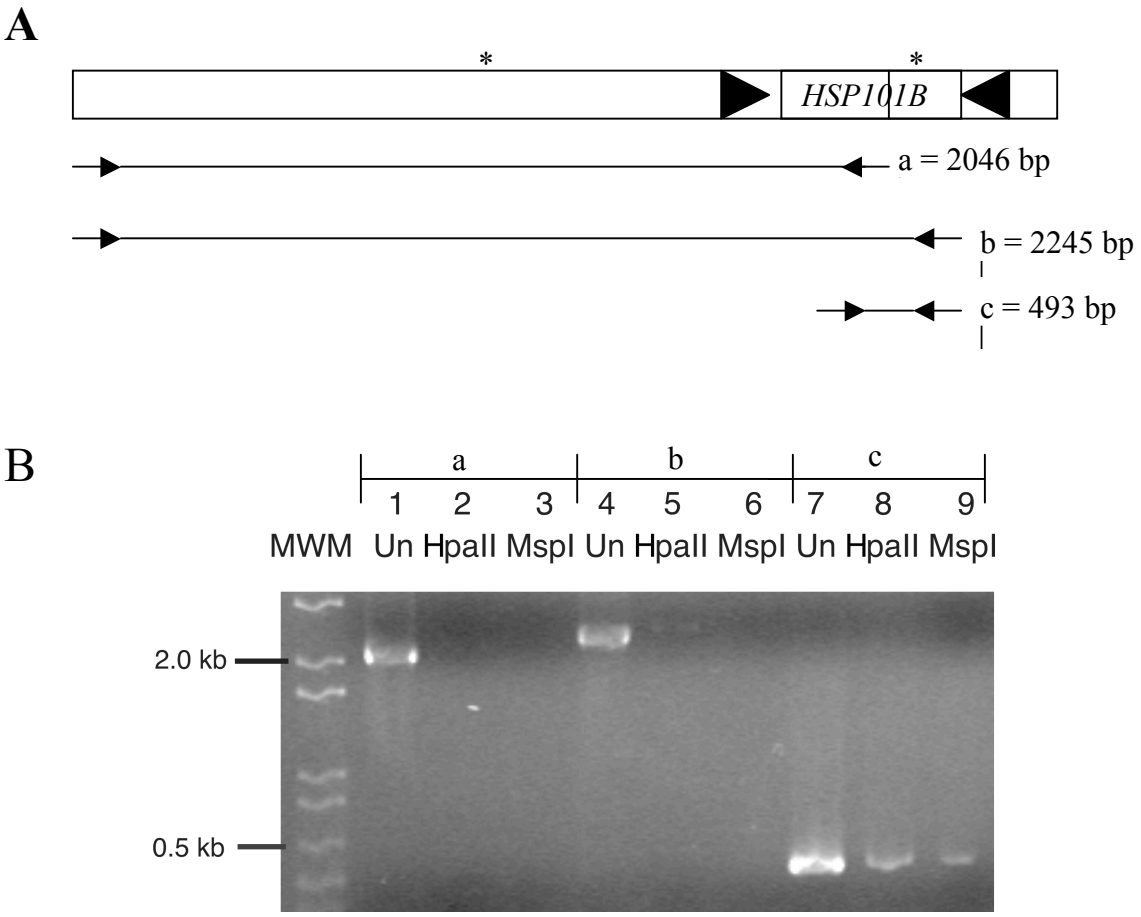


Figure 5.4 PCR amplification of *AtHSP101B* from undigested, HpaII and MspI digested *Arabidopsis* genomic DNA.
A) Diagram showing location of the 5'CCGG3' recognition sites for HpaII and MspI within *HSP101B* (*) and the primers used to amplify portions of *HSP101B* (arrows). The TIRs are denoted by black triangles and sizes of the fragments are given in bp. B) *A. thaliana* Columbia genomic DNA digested with HpaII, MspI or undigested (Un), amplified using the three sets of primers bracketing the *HSP101B* promoter (groups a and b, lanes 1-3 and 5-6, respectively) and the *HSP101B* ORF (group c, lanes 7-9). The *HSP101B* promoter is not methylated at the restriction enzyme sites, which lie outside the *MULE*, as evidenced by the lack of PCR product in the HpaII and MspI lanes (indicating the both restriction enzymes were able to digest the DNA). DNA inside the *MULE* is methylated (mCC) as both HpaII and MspI were unable to digest the DNA and PCR amplification was observed.

promoter activity is a reflection of the time when the *HSP101B* was a part of functional gene.

Transposition of promoter elements may be a means of generating genome-wide variation in promoter activity. In the case of *HSP101B*, a diametric change in activity was observed when the promoter was used in a transgene. Transposition of the *HSP101B* promoter (including the 5' upstream LTREs) into the promoter of an unlinked gene, or recombination between *HSP101B* and *HSP101,* could lead to both HTS and LTS inducibility for the resultant chimeric gene. Likewise, transposition of an ORF downstream of the *HSP101B* promoter could result in gene expression patterns similar to those observed in this study.

The ability of HSEs and LTREs to regulate transcription in response to temperature stress appears to be highly conserved across species. The *Arabidopsis HSP101B:GUS* transgene was active in both the distantly related *L. usitatissimum* and the more closely related *B. napus*. Furthermore, the previously described developmental activity of LTREs in *Arabidopsis* (Baker et al., 1994) was also conserved, with transcription observed in the flowers of both *L. usitatissimum* and *B. napus*. Developmental regulation of transgenic *HSP101B* transcription by the associated LTREs was also observed in the inflorescences of transgenic *Arabidopsis*. The endogenous *HSP101B* did not respond to LTS, however.

The *HSP101* promoter has at least five HSEs (Hong et al., 2001) whereas the *HSP101B* promoter only has one conserved and one partial HSE. The truncated *HSP101B* sequence 3' to the 5'TIR appears to be sufficient to induce transcription under HTS conditions, however. A T-DNA insertion 5' of the 4th HSE in the *HSP101* promoter did not prevent transcription although the insertion was sufficient to reduce seedling thermotolerance (Hong et al., 2001). I show here that transcription from *HSP101B* was induced under HTS conditions from only two HSEs although nearby *cis*-acting elements in the 5'TIR may have had some influence on this transcription.

Activity of the truncated *HSP101B* promoter may have been enhanced by the activity of the *MULE* promoter in the 5'TIR (Raizada et al., 2001). Activity from the 5'TIR promoter may explain the constitutive expression of GUS observed in some of the transgenic lines of flax, canola and transiently transformed *Arabidopsis*. Expression

from the endogenous *HSP101B* promoter was only HTS inducible, suggesting that promoter activity of the endogenous *MULE* 5'TIR promoter was not constitutive and that only the truncated *HSP101B* promoter was responsible for HTS-induced transcription. The expression patterns from the truncated *HSP101B* promoter (from 3' of the 5'TIR to the start codon) or the TIR itself were not determined. In future work the 5'TIR could be fused to Green Fluorescent Protein and the portion of the *HSP101B* promoter with similarity to *HSP101* fused to Yellow Fluorescent Protein. Activity of both promoters could be observed at the same time in transgenic plants containing these two constructs since the two fluorescent proteins could be visualized simultaneously using a confocal microscope.

If the transposition event that formed *HSP101B* was an ancient event I would expect there to be a low level of sequence conservation between *HSP101* and *HSP101B*. A high level of sequence identity was observed indicating possible sequence conservation and suggesting that *HSP101B* or its product may have some function in the cell. *HSP101B* may have a regulatory role in *HSP101* gene expression, possibly mediating temporary co-suppression of *HSP101* activity during HTS. Cosuppression of *HSP101* by *HSP101B* could be examined by producing transgenic plants overexpressing the *HSP101B* transcript (during HTS) or by mutating *HSP101B* itself.

HSP101 functions to help refold proteins denatured as a result of a high temperature stress (Lee et al., 1994; Nguyen et al., 1989; Schirmer et al., 1994). Under cold conditions protein denaturation may also occur due to changes in protein conformation directly either as a result of the low temperatures or because of low-temperature-induced changes in osmolyte concentration. *HSP101* expression was not induced by cold treatment in *Arabidopsis* (Hong et al., 2001), rice (Singla et al., 1998) or wheat. In contrast, four of the five *HSP70* genes in *Arabidopsis* (Li et al., 1999; Sung et al., 2001) and *HSP90* in *B. napus* (Krishna et al., 1995) were upregulated by cold induction. HSP70, HSP90 and HSP101 each has a different thermoprotective role.

Endogenous *HSP101B* was strongly transcribed only during HTS in *Arabidopsis*, but when the *HSP101B* promoter was used to control GUS expression in transgenic *Arabidopsis, B. napus* and *L. usitatissimum*, both constitutive and temperature stress-induced GUS activity were observed. Three possibilities could explain these

observations: I) control of *HSP101* expression in *L. usitatissimum*, *B. napus* and *Arabidopsis* is not conserved; II) positional effects silenced transcription of the endogenous *HSP101B* in *Arabidopsis* or; III) hypermethylation of the endogenous *HSP101B* prevented *HSP101B* promoter activity.

Transiently transformed *Arabidopsis* had the same *HSP101B:GUS* expression patterns as stably transformed *L. usitatissimum* and *B. napus* suggesting that control of *HSP101B* transcription is tightly regulated and conserved across species.

If silencing of the endogenous *HSP101B* occurred as a result of a nearby silencer then placing *HSP101B* in a new genomic environment, away from the influence of the silencing motif, would allow transcription to occur. *HSP101B:GUS* activity was observed in both transiently transformed *Arabidopsis* and stably transformed *B. napus* and *L. usitatissimum* but endogenous *HSP101B* transcripts were not observed in wild-type *Arabidopsis*. These observations support the hypothesis that silencing of endogenous *HSP101B* promoter activity is occurring.

Methylation is known to restrict transposition and/or activity of the *MuDR* TIR promoter (Bennetzen, 1996; Singer et al., 2001) and therefore methylation of the *MULE* TIR may down-regulate endogenous *HSP101B* transcription. Down regulation of the *HSP101B:GUS* transgene in transgenic plants would not be expected as PCR amplification and use of bacterial hosts during construction of the transgene would mean the transgene was hypomethylated.

The hypothesis that methylation plays a role in the regulation of endogenous *HSP101B* transcription is supported by my data. Endogenous *HSP101B* was methylated within the transposon (Fig 5.4), but the surrounding area of the genome appeared to be unmethylated. This methylation may have inhibited transcription of the *HSP101B* ORF from the 5'TIR in leaves under cold and control conditions (Fig 5.2). The *HSP101B:GUS* transgene, free of methylation in transgenic plants, was transcribed both constitutively and in response to LTS in stably transformed *B. napus* and *L. usitatissimum*. If methylation plays a role in suppression of endogenous *HSP101B* transcription, it would be of interest to see how the activity of the *HSP101B:GUS* constructs changed in transgenic plants over several generations as methylation of the originally unmethylated transgene occurred. Furthermore, it would be of interest to see

if LTS-induced and constitutive transcription of endogenous *HSP101B* occur in methylase mutants of *Arabidopsis* (Queitsch et al., 2002; Singer et al., 2001).

LTS of *Zea mays* led to the selective demethylation of *Ac/Ds* elements (Steward et al., 2000), while in *Medicago sativa*, LTS-induced retrotransposon activation, produced, no changes in methylation pattern (Ivashuta et al., 2002).

In this chapter I have shown that a *HSP101* gene fragment present in a *MULE* transposon still had transcriptional activity and produced truncated *HSP101* transcripts in HTS-treated *Arabidopsis* flower buds. Upstream of the promoter fragment are four LTREs, in various orientations, which are able to up regulate expression of the gene fragment under cold conditions and weakly control a specific developmental expression profile in the inflorescence. Methylation may play a role in the regulation of the *HSP101B* promoter. Endogenous *HSP101B* activity may have responded only to HTS and not LTS or constitutive cues due to methylation of the TIR. On the other hand, de-repression of *HSP101B:GUS* transgene expression in stably transformed *L. usitatissimum, B. napus* and transiently transformed *Arabidopsis* may have resulted from demethylation of the transgene. I suggest that transposition events resulting in gene rearrangements, such as the nonautonomous *MULE-24:HSP101* element, may be an evolutionary source of promoter function variability.

CHAPTER 6 GENERAL SUMMARY AND FUTURE WORK

Several aspects of the effects of HTS on plants during flowering and a means of overcoming these effects have been investigated in this work. I showed that HTS adversely affected both micro- and megagametophytes in *B. napus* resulting in an almost total absence of seed set. Knowledge of the physiological effects of HTS has major consequences for crop production not only in *B. napus* but also for other crops. Furthermore, understanding the physiological effects of HTS on flowering has offered potential targets for increasing thermotolerance via genetic manipulation. Several strategies for developing transgenic plants with improved thermotolerance were attempted. A better understanding of the physiology of HTS and the tissue affected by HTS might have improved the chances of developing plants with thermotolerant flowers. Further work to develop thermotolerant plants should take into consideration a range of HTS treatments, the promoters required to target sufficient gene expression in the tissues and developmental stages affected by HTS, regulation of the thermoprotective transgenes and the multitudinous effects of HTS on cell biochemistry. During the course of developing the transgenic plants I cloned and partially characterized a portion of *HSP101* that had been incorporated into a transposon. Interestingly, *HSP101B* was regulated by HTS and LTS possibly through changes in methylation level.

6.1 FUTURE WORK

Several aspects of this work could be continued in the future. An in-depth understanding of the physiological and biochemical effects of HTS on plant cells, especially the gametophytes, would not only improve our knowledge of the effects of HTS on plants but also suggest further targets and developmental stages to focus on. Improving the thermotolerance of these targets could lead to *B. napus* varieties with better yields in Saskatchewan. Furthermore, if a transgenic approach is used the

improvement of a variety of crops species could be investigated. Using a single (set of) transgene(s) that improved crop thermotolerance would also further our understanding of the effects of HTS in different species. Comparing the physiology of HTS or drought tolerant land races with *B. napus* varieties currently grown in Saskatchewan could also offer more insights into the mechanisms of thermotolerance and potentially be a source of thermoprotective transgenes.

Further experiments with the transgenic plants already developed are warranted. Investigating the effects of different degrees of HTS may indicate whether a small improvement in thermotolerance, not observable with the conditions used, was achieved by the transgenes. In addition, homozygous plants could improve thermotolerance by increasing copy number of the transgene in a cell. Another aspect of thermotolerance to investigate is vegetative thermotolerance and whether it has an impact on reproductive thermotolerance.

One experiment that could be repeated to gain an idea of basal thermotolerance is the reciprocal crosses of HTS and Control pollen and pistils. Performing this experiment under a variety of different HTS conditions could give a FLT_{50} based on the number of seeds produced by HTS pollen x Control pistils compared with Control pollen x Control pistil crosses. Using male sterile pollen receptor plants would eliminate the time consuming emasculation stage.

Two aspects of my work that did not have a direct application to my research goals are the regulation of *HSP101B* by methylation and the cosuppressive effects of orthologous *LFY* expression on *B. napus* flowering times. The observations reported here offer an interesting insight into the regulation of gene activity by mechanisms other than control of transcription and translation.

6.2 A FINAL CAVEAT

Improved knowledge of the effects of HTS on plant reproduction is beneficial as it increases our understanding of how plants react to abiotic stresses. The development of thermotolerant crops has potential drawbacks, however. Crops with increased thermotolerance, whether produced by conventional breeding techniques or by genetic engineering, have the potential to become weeds either through outcrossing with weedy relatives or by "escaping".

Outcrossing to a weedy relative, or otherwise transferring the thermotolerance trait, is possible only where crops and weedy relatives co-exist geographically. Transferal of thermotolerant traits to weedy relatives might not improve weed thermotolerance as the weedy relatives would likely already be adapted to the high temperatures experienced in the localities requiring the planting of thermotolerant crops. Outcrossing of the thermotolerance trait to a weedy species may allow the weed to extend it's geographical range, but this would only be possible if there was an ecological niche for which it might compete.

The thermotolerant trait may enable a crop species to "escape" by improving its ability to grow in a particular environment. Although possible, the chances of this happening are small. The escaped crops would only have a selective advantage during years of HTS. In years where HTS was not experienced, the energy costs of maintaining the thermotolerance trait may be a selective disadvantage. Furthermore, a single trait is unlikely to turn a crop species into a weed. A number of traits need to be modified before most crop species become weedy.

In the current socio-political environment there is concern that transgenic species with improved tolerance to abiotic stresses could become "superweeds". There is some risk that plants with greater tolerance to abiotic stresses could become problems; however, the risk is the same whether the plant is transgenic or the result of "traditional" breeding. Arguments against the release of stress-tolerant plants should not be based on the technique used to obtain the plants but on the trait itself.

CHAPTER 7 REFERENCES

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